Effect of low frequency ultrasound and ultraviolet-C light for water disinfection in recirculating aquaculture systems

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You are only here for a short visit. Don't hurry, don't worry, and be sure to smell the flowers along the way.

Walter Hagen

This thesis is dedicated to my lovely wife Maryam and our dear son, Rayan.

Zusammenfassung

In der Aquakultur sind Kreislaufanlagen (recirculating aquaculture systems, RAS) ein umweltfreundliches und wassersparendes Produktionsverfahren. Hohe Besatzdichten und das Prinzip der Wasserführung im Kreislauf führen jedoch auch zu einem erhöhten Risiko von Infektionskrankheiten. In dem hier beschriebenen Projekt wurde untersucht, wie sich niederfrequenter Ultraschall (nf-US) in Kombination mit der schon in der Aquakultur bewährten UV-C Bestrahlung einsetzen lässt.

Es wurden vergleichende Untersuchungen zur Effizienz von nf-US, UV-C und deren Kombination gegen prokaryotische und eukaryotische Modellorganismen durchgeführt und die dosisabhängigen Eliminationsraten bestimmt. Als Modellorganismen dienten heterotrophe Bakterien (Gesamtkeimzahl) und eukaryotische Organismen, die Taxa verbreiteter Fischparasiten repräsentieren: der Ciliat *Paramecium* sp., das zweite Larvenstadium (L2) des Nematoden *Anguillicola crassus* und Metanauplien von des Crustaceen. *Artemia* sp. Außerdem wurde der Effekt von nf-US auf frei schwimmende *Trichodina* sp., einem fischpathogenen Ciliaten, untersucht.

Während sich UV-C als sehr effektiv gegen Bakterien erwies, konnte die Gesamtkeimzahl mit nf-US mit bis zu 19 kJ/L bei einer einmaligen Passage des Reaktors nicht reduziert werden. Eine Vorbehandlung des Wassers mit nf-US verringerte die mittlere Größe der im Wasser einer Kreislaufanlage suspendierten Partikel und konnte so die Effektivität von UV-C zur Inaktivierung von Bakterien um bis zu 0,6 log-Einheiten verbessern. Im Kreislaufbetrieb konnte die Gesamtkeimzahl mit UV-C innerhalb von 96 h signifikant reduziert werden, wenn der gesamte Wasserstrom (133 % des Anlagenvolumens pro h) behandelt wurde. Die Abnahme der Gesamtkeimzahl erfolgte jedoch deutlich langsamer als dies entsprechend einem mathematischen Modell mit den Annahmen eines exponentiellen Wachstums in Kombination mit einer linearen Elimination zu erwarten gewesen wäre.

Im Bypassbetrieb (67 % des Anlagenvolumens pro h) konnte auch mit einer nf-US Vorbehandlung keine Reduktion der Gesamtkeimzahl erreicht werden. Dies zeigt, dass die Reduktion von heterotrophen Bakterien in Kreislaufanlagen eine hohe Umsatzrate erfordert, um deren kurze Generationszeit zu kompensieren.

Gegen die eukaryotioschen Organismen erwies sich nf-US als wirksam, wobei die dosisabhängige Abtötung sehr gut mit Funktionen einer exponentiellen Abnahme beschrieben werden konnte. Gleichwohl unterscheidet sich die unterschiedlichen

Organismen stark in ihrer Empfindlichkeit gegenüber nf-US. Die einmalige Anwendung einer nf-US Dosis von 1,9 kJ/L (spezifischer Energieverbrauch) reichte aus, um für *Artemia* Metanauplien eine Reduktion um 99 % zu erzielen, während eine zehnfach höhere nf-US Dosis erforderlich war, um *Paramecium* sp. und *A. crassus* Larven um 95 % bzw. 81 % zu reduzieren.

Im Kreislaufbetrieb konnten frei schwimmende *Trichodina* sp. mittels nf-US innerhalb von 96 h signifikant reduziert werden. Hierbei folgte die gemessene Abnahme der frei schwimmenden Parasiten einem mathematischen Modell mit den Annahmen eines exponentiellen Wachstums in Kombination mit einer linearen Elimination; allerdings erfolgte die tatsächliche Abnahme der Parasiten langsamer als dies nach dem Modell zu erwarten gewesen wäre.

In Wasser mit einem geringen spektralen Schwächungskoeffizienten bei 254 nm (SSK_{254}) erwies sich UV-C (emittiert von einer Niederdrucklampe) gegenüber *Paramecium* sp. und *A. crassus* im Vergleich zu nf-US als das energetisch effizienter, während nf-US das effizientere Verfahren gegen *Artemia* sp. war. Gleichwohl wäre die Effizienz von nf-US gegen Ciliaten oder Nematodenlarven ähnlich oder sogar besser als die Effizienz von UV-C, wenn das Wasser einen hohen SSK_{254} aufweist und/oder wenn die weniger effizienten UV-Mitteldrucklampen verwendet werden.

Die toxikologische Untersuchung des mit UV-C und/oder nf-US behandelten Wassers mit dem Fischeitest und dem Leuchtbakterientest ergab keinen Hinweis auf die Bildung toxischer Nebenprodukte. Gleichwohl kann die Option, die UV-C Dosis zur Abtötung eukarytotische Pathogene weit über die zur Kontrolle von Bakterien empfohlene Dosis zu erhöhen, durch die photo-induzierte Bildung von Nitrit aus Nitrat eingeschränkt sein, da Nitrit stark toxisch auf Fische wirkt. Alternativ könnte nf-US zur Abtötung eukariotischer Parasiten verwendet werden. Es handelt sich hierbei um ein sicher anwendbares Verfahren, das eine sinnvolle Ergänzung zu der gegen Bakterien verwendeten UV-C-Strahlung sein kann.

In der Praxis kann in RAS ein kombinierter UV-C/nf-US Reaktor eingesetzt werden. In einem kombinierten Reaktor bestimmt die UV-Einheit die erforderliche Durchflussrate. Daher sollte ein solcher Reaktor mit dem vollen Volumenstrom des Systems beaufschlagt werden und könnte in kleineren Anlagen direkt in den Filterkreislauf eingebaut werden. Bei hohen Durchflussraten großer Kreislaufanlagen kann es unter technischen und energetischen Gesichtspunkten günstiger sein, separate UV-C und nf-US Reaktoren zu verwenden. Dann sollte der UV-C-Reaktor mit einer geringen, zur

Reduktion der Gesamtkeimzahl ausreichenden Dosis betrieben und mit dem gesamten Volumenstrom des Filterkreislaufes beaufschlagt werden, während der nf-US-Reaktor zu Bekämpfung eukaryotischer Parasiten im Bypass verwendet werden kann.

Diese Studie zeigt, dass nf-US mit Dosen, die gegen eine Vielzahl an Parasiten wie Ciliaten, Nematoden und Crustaceen wirksam sind, sicher eingesetzt werden kann. Die Kombination von nf-US und UV-C könnte ein angemessenes Verfahren zu Wasserbehandlung in RAS sein, um alle relevanten Pathogene zu kontrollieren.

Summary

Recirculating aquaculture systems (RAS) are well-known to high water-efficient production processes. The highest available stocking densities and low water exchange lead to an increased risk of infectious diseases. In this project, a disinfection strategy involving low frequency ultrasound (LFUS) and Ultraviolet–C (UV-C) light was investigated in a sole or combined mode. Comparative studies on the efficiency of LFUS, UV-C and their combined application against prokaryotic and eukaryotic model organisms were performed. Dose-dependent reduction rates were determined for bacteria and eukaryotic model organisms representing different taxa of common fish parasites: the ciliate *Paramecium* sp., second larval stage (L2) of the nematode *Anguillicola crassus* and metanauplii of *Artemia* sp. and also free-swimming *Trichodina* sp. as a real ciliated ectoparasite.

Application of LFUS in a single-pass mode up to 19 kJ/L did not reduce the number of total viable count (CFU/mL), whilst UV-C irradiation was highly effective. Pre-treatment with LFUS reduced the mean size of suspended solids in RAS-derived water and thus increased the germicidal effect of UV-C by up to 0.6 log units. In continuous-pass mode, the full-flow application of UV-C in RAS (133 % of RAS water volume per h) significantly reduced the bacterial count within 96 hours. Despite the significant reduction of bacterial count by continuous application of UV-C in full-flow mode, the course of the measured bacterial reduction was much slower than the prediction by mathematical model assuming the exponential growth and a linear reduction of organism in circulating water.

In contrast, UV-C application in a bypass with 67 % of RAS water volume per h, even with Pre-treatment with LFUS did not reduce the bacterial count which proving that the reduction of heterotrophic bacteria in RAS requires a high turnover rate in order to compensate their short generation time.

LFUS was effective against the eukaryotic organisms, and the dose-dependent reduction could be well described by functions of an exponential decay. However, the efficiency of LFUS differed greatly between species. A single application of LFUS with consumed specific energy of 1.9 kJ/L was sufficient to reduce *Artemia* sp. by 99 %, but a ten times higher dose was necessary to reduce 95 % and 81 % of *Paramecium* sp. and *A. crassus* larvae, respectively.

The continuous application of LFUS in a bypass mode resulted in a significant reduction of free-swimming *Trichodina* sp. in RAS within 96 hours. The course of the measured reduction of free-swimming *Trichodina* sp. followed the mathematical model, but proceeded more slowly than the predicted reduction.

In water with low spectral attenuation coefficient (SAC_{254}), the energetic efficiency of UV-C (emitted by a low pressure lamp) against *Paramecium* sp. and *Anguillicola crassus* larvae was higher compared to LFUS, but LFUS was more efficient against *Artemia* sp. However, the efficiency of LFUS against ciliates or nematode larvae would be similar or even higher than UV-C in water with high SAC_{254} and/or if less efficient medium pressure lamps are used.

The evaluation of whole effluent toxicity by fish egg test and Luminescent bacteria test revealed no evidence of toxic disinfection by-products formation during UV-C irradiation and/or LFUS sonication. However the potential to increase the UV-C dose against eukaryotic parasites, much higher than the recommended UV-C dose for reduction of the bacteria, might be limited by the photoinduced formation of nitrite from nitrate which is harmful to fish. Alternatively, LFUS can be used for reduction of eukaryotic parasites. The application of LFUS can be used as a suitable and safe-applicable method for eliminating eukaryotic parasites and can thus be a useful supplementation to UV-C used against bacteria.

In practice, a combined UV-C and LFUS disinfection reactor can be applied in RAS. In a combined reactor, the UV-unit determines the necessary flow rate. Thus, this reactor should be operated continuously with the full-flow of the system. In small RAS it could be installed directly in the filter circuit. At high flow rates, it may make more sense from a technical and energetic point of view to use separate UV-C and LFUS disinfection reactors. In this situation the UV-C disinfection reactor should be charged with the full volumetric flow of the system at typically recommended dose in aquaculture to reduce the bacterial load and the LFUS disinfection reactor can then be operated in the bypass mode for the reduction of eukaryotic parasites.

This study shows that LFUS can be applied safely at energy densities that are effective against a wide range of eukaryotic parasites like ciliates, nematodes and crustaceans. The combination of LFUS and UV-C could provide an appropriate water treatment with regards to all relevant pathogens in recirculating aquaculture systems.

Schlagwörter: Niederfrequenz-Ultraschall, Ultraviolet-C, Desinfektion, Geschlossene Kreislaufanlagen, Nitrit

Keywords: Low frequency ultrasound, Ultraviolet-C, Disinfection, Recirculating aquaculture system, Nitrite

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RAS	Recirculating Aquaculture Systems
UV-C	Ultraviolet-C
LFUS	Low Frequency Ultrasound
SAC	Spectral Attenuation Coefficient
DOM	Dissolved Organic Matters
TSS	Total Suspended Solids
LP-Lamp	Low Pressure Lamp
MP-Lamp	Medium Pressure Lamp
AOP	Advanced Oxidation Process
LD	Laser Diffractometry
WET	Whole Effluent Toxicity
FET	Fish egg test
CFU	Colony forming unit
DPH	Day Post Hatch
L2	Second larval stage
DAPI	4', 6-diamidino-2-phenylindole

pH	measure of acidity/basicity of aqueous solutions
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
Disinfection by-products	DBPs
Public water systems	PWSs
3,4-DCA	3,4-Dichloroaniline

1 Introduction

1.1 Problem statements

Aquaculture is considered as the fastest growing form of agriculture all-around of the world, with an average annual growth rate of 8.9 % since 1970, as compared to capture fisheries with 1.2 % and terrestrial meat production with 2.8 %, (Subasinghe, 2005). Due to the global water scarcity (Vörösmarty et al. 2010), growth of the land-based aquaculture mostly depends on increasing the stocking densities in a given volume of the water than increasing the culture area (Avnimelech et al. 2008). One of the best examples of intensive aquaculture systems are recirculating aquaculture systems (RAS), which can efficiently reduce the overall water consumption and improve the control of nutrition, water quality and biosecurity (Yanong 2012). In RAS, the most important principle is the reuse of running water in an appropriate way and any adverse change in the water quality often causes stress, decreased product quality and furthermore endangers the health of the fish. Thus, attention to the health condition of RAS poses specific challenges in the case of water quality and disinfection. RAS with the high stocking densities and a great potential of pathogen accumulation are very susceptible to disease outbreaks (Martins et al. 2010). Disease outbreaks caused by pathogens are one of the most important limiting factors that significantly affect the socioeconomic development of aquaculture (Bondad-Reantaso et al. 2005). In a sustainable and profitable aquaculture industry, attention to the aquatic animal's health and the reduction of losses caused by disease outbreaks are key managing factors. The control and reduction of pathogens in production systems can be achieved by sanitation and disinfection processes. Disinfection, or destroying of pathogenic microorganisms, can be obtained by means of chemical or physical methods (Yanong and Erlacher-Reid 2012). In an intensive aquaculture system with high stocking densities, the fish farmers mostly rely on the use of chemicals for the prevention and treatment of the diseases during the culture period (Burridge et al. 2008). The public concern about human health, food safety and environmental impacts has resulted in an increasing attention and enforcement regarding the use of chemicals (Reilly and Käferstein 1997). In the aquaculture industry, the approval process of a new chemical compound requires the safety and efficacy experiments which require much time and investment. Therefore, the development of the aquaculture industry is much faster than the development of regulations and approval processes for new chemical compounds. On

the other hand, a great public concern can be considered as a limiting factor of chemical application in aquaculture, from the point of view of residuals and environmental impact (Costello et al. 2001). However, the lack of approved drugs and chemicals has dramatically reduced the effectiveness and increased the cost of production in the aquaculture industry (Schnick 1996). Compared to the number of commercially available chemicals for treatment and disinfection aims, the physical disinfection strategies in RAS are mostly limited to the application of ultraviolet-C (UV-C) light (OIE 2003). Thus, the development of an environmentally-friendly and non-chemical disinfection strategy is becoming increasingly important in the aquaculture industry.

1.2 Statement of the technique

Attention to water quality by means of water disinfection can control the spread of pathogens and prevent disease outbreaks. The application of ozone and UV-C light are the most common disinfection techniques used in aquaculture today (Summerfelt 2003). Ozone can both disinfect and improve the quality of the water in its role as a powerful oxidizing agent in aquaculture. In water with high organic load, the half-life of ozone is significantly reduced, and thus superb disinfection efficiency requires the application of higher doses (Bullock et al. 1997). High toxicity and the risk of malfunction are other concerns of ozone application which may cause serious harm to the operator and the fish stocks (Summerfelt 2003). Therefore, attention to the safety requirements of the ozone application in RAS calls for highly technical efforts and considerations, which can increase the cost of the production (Jorquera et al. 2002).

The irradiation of water with UV-C is a physical method commonly used in aquaculture and plays an important role in water disinfection processes. Inside aquaculture facilities, UV-C is used mostly for the prevention of bacterial, viral and fungal diseases (Kasai et al. 2002). UV-C, at a wavelength of 254 nm, most effectively denatures the genetic materials (DNA and RNA) of microorganisms, causing a reduction in their numbers by preventing the microorganisms from replicating (U.S.EPA 2006). The efficiency of UV-C depends on the light transmittance of the treated water which is adversely affected by strong light absorption by dissolved organic matter (DOM) and light scattering by total suspended solids (TSS) (Gullian et al. 2012; Liu and Zahng 2006). Therefore, the efficient application of UV-C requires a low concentration of dissolved and suspended matters, conditions that are not always economically and practically available in RAS (Gullian et al. 2012).

Another potential physical disinfection method not impaired by water turbidity (Gibson et al. 2008) and with a good potential to reduce organisms larger than approximately 100 µm is low frequency ultrasound (LFUS) (Holm et al. 2008). Ultrasound, with a frequency of 20 kHz or above, generates cavitation phenomena that can be used for disinfection purposes (Gogate 2007).

Most previous studies on the disinfection efficiency of cavitational reactors were conducted with a long exposure time of up to several minutes, and were targeted against bacteria primarily, with hardly any focus on eukaryotic pathogens (Scherba et al. 1991: Gogate 2007). High flow rates in flow-through disinfection reactors result in short exposure times only compared to exposure times applied in previous laboratory studies. Furthermore, the method should provide an appropriate reduction rate of all relevant pathogens including prokaryotic and eukaryotic parasites. Finally sonication has the potential to be applied to a diverse range of water disinfection processes used either alone or, more commonly combined with other common disinfection methods such as UV-C light (Blume and Neis 2004). The application of a flow-through cavitational reactor for water disinfection in RAS is not studied, but recently the combination of LFUS and UV-C was examined for waste and ballast water treatment (Naddeo et al. 2009; Sassi et al. 2005). The combination of LFUS and UV-C processes can be considered as a novel and innovative approach for the management and optimization of fish health without the use of unhealthy and environmentally harmful chemicals or medications.

1.3 The scopes and objectives of the study

RAS with their high risk for pathogen accumulation and consequent disease outbreaks are the target systems for the application of innovative LFUS disinfection technology. The aims of this study are the development of an efficient and industrially applicable disinfection process for RAS. The disinfection system should allow the simultaneous treatment of the circulating water with LFUS and UV-C in a continuous flow–through operating system. This study assesses the application of LFUS and UV-C light in the reduction of a wide range of prokaryotic and eukaryotic pathogens commonly present in RAS. The project comprises the following steps:

1) Application of LFUS and UV-C against different model organisms and proof of reduction rate in a single-pass mode

2) Investigation of sole and combined effect of LFUS and UV-C light in a continuous mode and definition of essential process parameters

3) Investigation of possible disinfection by-products in a sole and combined application mode of LFUS and UV-C light which could affect the health of the fish

2 Literature review

Efficiency is the most important issue in water disinfection by means of chemical and physical strategies. A number of chemical and physical approaches have been considered for water disinfection in the aquaculture industry. For example, most chemicals widely used as a sanitation and disinfection agents cause the formation of non-acceptable disinfection by-products (DBPs) (Sonntag and Schuchmann 1992; Costello et al. 2001). In addition to a great concern about the environmental and health issues, the efficiency of chemical agents will be affected when the water contains high amounts of total suspended solids (TSS) which can be considered as refuge for microorganisms such as bacteria. The particulate maters in water entrap the microorganisms and the chemical treatment of these colonies may destroy the surface microorganisms and leaving the inner and embedded organisms intact (Gogate 2007) (Fig. 1).

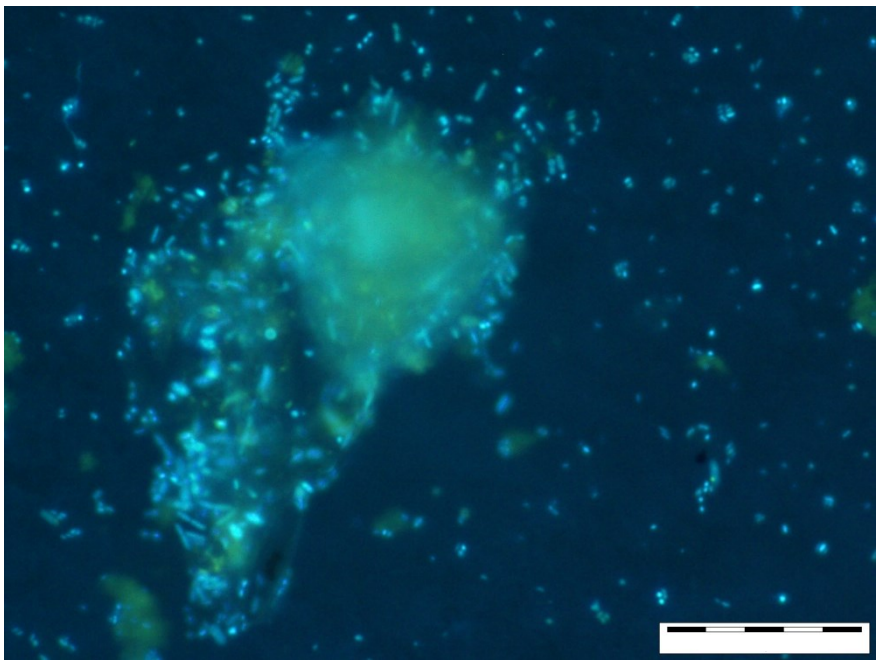

Figure 1. DAPI stained colony of heterotrophic bacteria attached to suspended particles in RAS-derived water. Scale bar: 20 µm.

The potency of physical techniques, such as UV-C as the most common physical disinfection strategy (Kasai et al. 2002), is also limited in high light scattering and absorbing environments such as RAS (Guilan et al. 2012). In these systems, the vast majority of suspended particles can be removed by physical filtration systems, but the

dissolved organic matter and small suspended particles still remain inside the water. Thus, there is a need for developing an alternative technique that meets the requirement of appropriate water disinfection and compensates the limitations of UV-C light. LFUS offers the possibility to be used as an effective and novel method for water disinfection due to its high potential in terms of hot spot generation, highly reactive free radicals and turbulence (Gogate 2007).

2.1 Ultraviolet-C (UV-C) light

Ultraviolet light is an electromagnetic wave between X-rays and visible light (Fig. 2) with a wavelength of 100 - 400 nm including vacuum UV (100 - 200 nm), UVC (200 - 280 nm), UVB (280 - 315 nm) and UVA (315 - 400 nm) (U.S.EPA 2006).

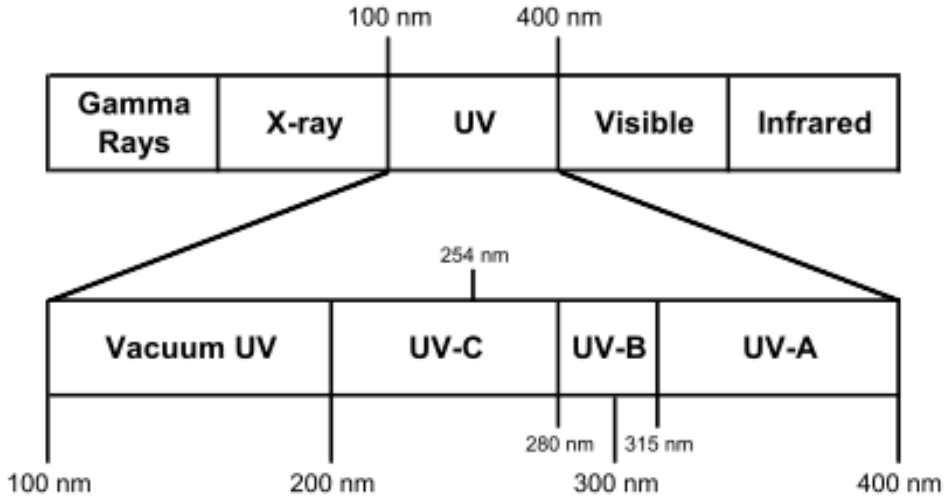

Figure 2. Ultraviolet light in the electromagnetic spectrum (U.S.EPA 2006).

The light emitted at the wavelength of 254 nm is well absorbed by DNA of the target organism and leads to a destruction of the DNA structure, thus preventing the microorganism from replicating (Liltved 2002). For disinfection and germicidal purposes, UV irradiation between the wavelength of 240 and 280 nm (UV-C) is mostly applied (U.S.EPA 2006). Reduction can be also achieved at other UV wavelengths from 100 to 400 nm, although a wavelength of 254 nm is most effective (Sharrer et al. 2005). The first application of UV light was based on the disinfection process of drinking water facilities in the beginning of the twentieth century, with a huge increase in the global application of ultraviolet light in waste and drinking water industry occurring afterward (Whitby and Scheible 2004).

In the aquaculture industry, especially in RAS, UV-C irradiation is also considered as an appropriate disinfection technology (Liltved 2002; Sharrer et al. 2005; Blancheton 2000). Low pressure lamps (LP-lamps) and medium pressure lamps (MP-Lamps) are the most common lamps used for disinfection purposes. LP-Lamps are almost monochromatic, with UV output at a single wavelength of 254 nm, but MP-Lamps are polychromatic, with UV output from 200 to 320 nm (Fig. 3). Both LP and MP-Lamp systems perform equally well in reducing the numbers of microorganisms, but each has distinct advantages in different applications. Compared to MP-Lamps, LP-Lamps have approximately 3 times higher germicidal efficiency, smaller power draw per lamp and longer lamp life (U.S.EPA 2006) and are more common for water disinfection in RAS. MP-Lamp systems, due to higher intensity, have a much greater treatment capacity compared to LP-Lamp systems (approximately 25 times) (Wolfe 1990). MP-Lamps are also available, but not as commonly used in the aquaculture industry (Summerfelt 2003).

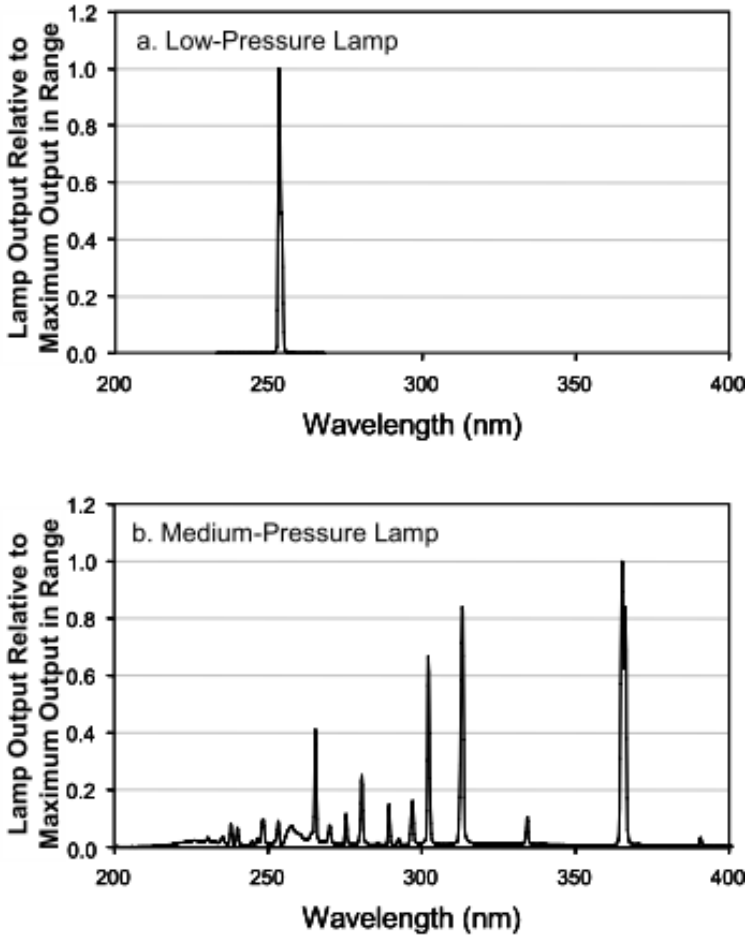

Figure 3. UV output of low pressure (LP) (**a**) and medium pressure (MP) (**b**) Mercury Vapor Lamps. (U.S.EPA 2006).

In order to quantify the bacterial reduction by UV-C light, the applied dose has to be calculated by the following formula (U.S.EPA 2006):

$$D = I \times T$$

where (D) is the UV dose in mWs/cm^2, (I) is the light intensity in mW/cm^2 and (T) is the exposure time in S.

The typical bactericidal dose of UV-C for a specific water flow in RAS is normally around 30 mWs/cm^2 (Sharrer et al. 2005; Guilan et al. 2012). In practice, the performance of UV-C efficiency is negatively influenced by water quality in terms of high water turbidity (Liltved 2002; Gulian et al. 2012). The commercial RAS with high stocking densities have high amounts of dissolved and suspended solids. These absorbing and scattering environments negatively affect the efficiency of UV-C in the reduction of free living and embedded bacteria (Sharrer et al. 2005; Guilan et al. 2012).

The formation of DBPs may occur during UV disinfection of water. The production of undesirable DBP_S during UV irradiation has been a matter of concern in several studies, as these DBP_S may violate safety requirements and corresponding drinking water regulations (U.S.EPA 2006). One of these DBPs is nitrite (NO_2^-) and the photoinduced formation of NO_2^- from nitrate (NO_3^-) in public water systems (PWSs) has been studied (Buchanan et al. 2006; IJpelaar et al. 2005; Lu et al. 2009; Sharpless et al. 2003). The photochemistry of NO_2^- and NO_3^- was well described (Mack and Bolton 1999). In aqueous solutions, NO_3^- has a strong absorption in the lower UV spectrum below 230 nm with a maximum at 200 nm, and a weak absorption with a maximum at 300 nm. NO_3^- photolysis leads to the formation of NO_2^- and oxygen (Mack and Bolton 1999; Takeda and Fujiwara 1993). The level of NO_2^- formation depends strongly on the UV dose and the ambient NO_3^- concentration (Mack and Bolton 1999; IJpelaar et al. 2005; Lu et al. 2009; Sharpless et al.2001; Sharpless et al. 2003) and rises with increasing pH (Mack and Bolton 1999; Lu et al. 2009).

Inside drinking water facilities, NO_2^- formation is not significant when LP lamps are used (IJpelaar et al. 2005) but MP lamps can cause a much higher NO_2^- formation due to a much stronger emission at wavelengths between 200 and 240 nm, where NO_3^- absorbs strongly (IJpelaar et al. 2005; Sharpless et al. 2003, Summerfelt 2003). However, even for LP-lamps, the application of hydrogen peroxide, as well as alkaline

conditions and uncommonly high UV doses can result in a nitrite yield that exceeds the common drinking water standard of 1 mg/L (Lu et al. 2009; Sharpless et al. 2003). In RAS, nitrate as the end product of the nitrification process (Pillay and Kutty 2005) can easily reach several hundred milligrams per liter (Van Bussel et al. 2012) which is much higher than in the drinking water industry.

LP lamps mostly emit light at 253.7 nm, the wavelength that accounts for the highest germicidal effect (U.S.EPA 2006). However, LP-lamps possess further emission lines at 184.9, 313.1, 365.0, 404.7 and 435.8 nm, altogether contributing about 10 % to the total emitted energy (Roig et al. 1999). Radiation situated below 240 nm has a pronounced photochemical effect and is therefore prohibited from use in UV radiation sources for disinfection of drinking-water (Figawa 2009).

The use of doped quartz glass in ozone-free lamps, filters out the 185 nm UV radiation that is responsible for ozone production, but it cannot prevent the emission of wavelengths above 240 nm. Considering the absorption spectrum of NO_3^-, it becomes obvious that the excitation of NO_3^- at 253.7 nm and 313.1 nm, both wavelengths emitted by LP lamps, could promote the reduction of NO_3^- to NO_2^- (Mack and Bolton 1999; Takeda and Fujiwara 1993). Therefore, photoinduced formation of NO_2^- from nitrate NO_3^- by UV-C irradiation might pose a serious health risk for the fish because nitrite adversely affects the oxygen-carrying capacity of blood by changing hemoglobin to methemoglobin (Lewis and Moris 1986).

In addition to the acute toxicity, sub lethal effects of NO_2^- on the fish such as physiological disturbances, tissue damage and reduced growth (Alcaraz and Espina 1997; Frances et al. 1998; Kroupova et al. 2008; Wuertz et al. 2013) must be also considered.

The application of UV-C in PWS_s is mostly against prokaryotic organisms which can be effectively reduced by using the recommended UV-C dose of 40 mWs/cm^2 (Kolch 2007). However, the efficiency of UV-C light against the eukaryotic pathogenic organisms is not fully studied. According to the literature, a reduction of eukaryotic microorganisms calls for higher ultraviolet energies compared to prokaryotic organisms (Gratzek et al. 1983; Hoffmann 1974; Colorni and Burgess 1997). Thus, UV-C irradiation by LP-lamps in RAS with high nitrate content might become critical when applied in a much higher dose than the recommended dose for bacterial reduction.

As such, the photoinduced formation of nitrite might limit the use of higher UV doses that are required for the reduction of eukaryotic parasites. However, there are no published data on whether the use of LP lamps in RAS could result in a critical, toxic nitrite formation. Due to the above-mentioned information, it seems that the sole application of UV-C light is not the optimal choice for the total reduction of a vast range of pathogens in RAS, requiring then a compensatory disinfection method to overcome the limitations of UV-C.

2.2 Low frequency ultrasound (LFUS)

Ultrasound, with a frequency of 20 kHz or above, is beyond the limits of human hearing. According to frequency, ultrasound is divided into three categories including low frequency ultrasound (LFUS) having a frequency of 20 - 100 kHz, high frequency ultrasound having a frequency of 0.1 - 1 MHz and diagnostic ultrasound having a frequency of 1 - 500 MHz (Wu et al. 2013).

LFUS is used in chemically important systems in which chemical and physical changes are desired, as it has the ability to cause cavitation phenomena. Ultrasound ranging from 1 to 10 MHz is used for animal navigation and communication, detection of cracks or flaws in solids, underwater echo location, and for diagnostic purposes (Pilli et al. 2011) (Fig. 4).

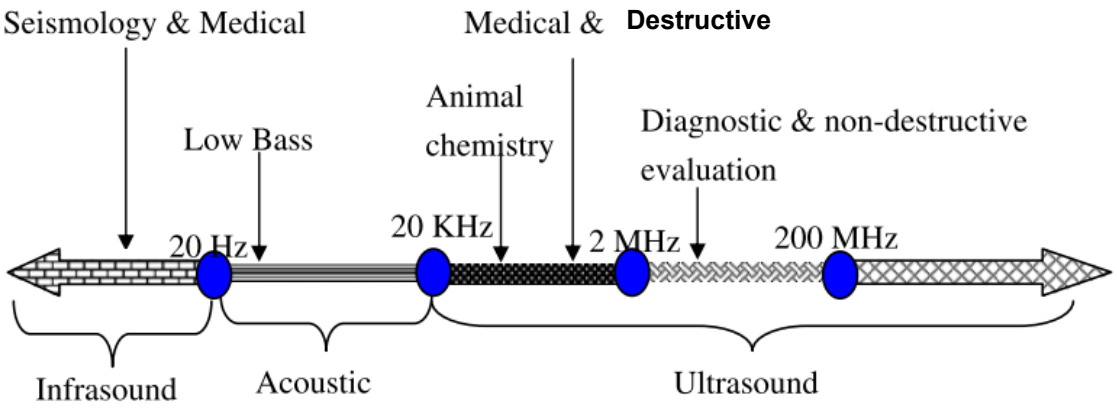

Figure 4. Diagram of ultrasound range (Pilli et al. 2011).

The application of ultrasound in aqueous solutions can produce cavitation phenomena, which is defined as the formation, growth and subsequent collapse of micro bubbles (Fig. 5).

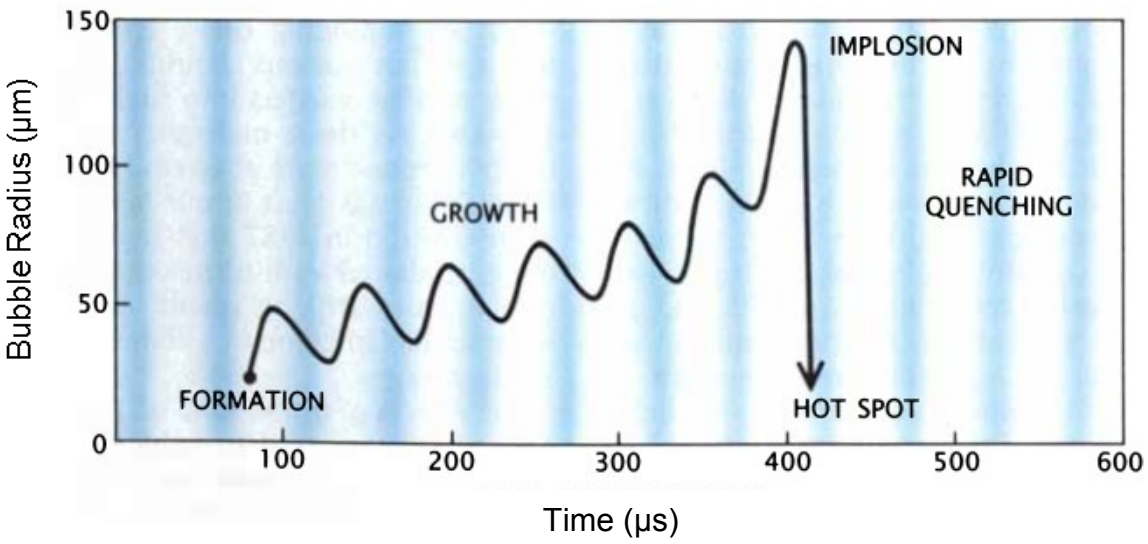

Figure 5. Formation, growth and implosion of cavitation bubbles in aqueous solution under ultrasonic irradiation (Suslick 1990).

Ultrasonic technology has received wide attention in wastewater treatment and environmental issues during the past years. Sonication has been effectively applied as an advanced oxidation process (AOP) against a wide variety of pollutants in the wastewater industry because it does not need the addition of oxidants or catalysts, and does not generate additional DBPs as compared to adsorption or ozonation processes (Wu et al. 2013). However the production of cavitation caused by higher ultrasonic frequencies becomes more difficult than low ultrasonic frequencies (Adewuyi 2001) and it makes the LFUS a better candidate for water disinfection than high frequency ultrasound. The formation, growth and collapse of cavitation bubbles which are shown in Fig. 5 create high-energy chemical reactions (Sonochemical effect) due to enormous local temperatures and pressure (Suslick 1990) and also a significant physical effect (sonophysical effect) (Wu et al. 2013) that offers potential as an effective tool for water disinfection (Gogate 2007).

Degradation of particulate matters by sonication is a non-random process, with the breakup taking place roughly at the center of target particle. Larger particles with larger mass are more susceptible to the ultrasonic treatment (Grönroos et al. 2008). Due to the

sheltering effect of particles for micro-organisms, the application of ultrasound can also be considered as a potential de-agglomeration method to reduce the size of particles as preparation of a suitable substrate for another disinfection strategy such as UV-C light irradiation (Blume and Neis 2002).

The exact mechanism by which cavitation can result in reduction of the microorganisms leading to water disinfection has not been conclusively established, though it is a combination of mechanical, thermal and chemical mechanisms (Thacker 1973; Doulah 1977; Mason et al. 2003). It has been generally observed that the mechanical effects are more responsible for the microbial disinfection and that the chemical and heat effects play only a supporting role (Mason et al. 2003).

Compared to the application of LFUS as a disinfection strategy in waste and ballast water treatment, the application of LFUS in aquaculture is limited to one study in which the fish mortality was controlled by ultrasonic reduction of infective stages (cercariae) of *Bucephalus polymorphus* in input water to the fish pond (Wolber and Pietrock 2004).

It seems that the application of LFUS as a novel technology has the potential to meet the sanitation requirements of RAS and can be considered as a good disinfection alternative to the conventional chemicals and the use of sole application of UV-C light.

The application of LFUS in RAS with continuous flow-through mode and high flow rate needs a special design and technical optimization. The flow-through LFUS disinfection reactor should provide an appropriate disinfection efficiency for a short exposure time and also should have the potential for sole or combined application with other conventional physical disinfection methods such as UV-C light.

3 Materials and methods

3.1 Experimental disinfection reactors

3.1.1 LFUS disinfection reactor

In the present study we used a LFUS disinfection reactor (Vortex reactor WR 4-1402.03). The technical specifications of this novel disinfection reactor are summarized in Table 1. The design and construction of the innovative LFUS disinfection reactor was done by Bandelin electronic, Berlin, Germany as project partner. The construction design of the LFUS disinfection reactor (Fig. 6) is characterized by four rows of transducers that are externally mounted on the disinfection reactor tube. The ultrasonic intensity rapidly decreases both radially and axially from the ultrasonic transducer (Santos et al. 2009). To obtain the minimum dead zone in sonication area, the space between the water and the internal wall of the disinfection reactor tube must be kept to a minimum. The targeted rotary movement of the sonication medium provides cavitation intensive flow-through sonication in a narrow reaction gap all-around of the disinfection reactor cylinder. The volume-specific power of the LFUS disinfection reactor was adjustable in 10 % steps within a power density ranging from 48 to 480 W/L.

Table 1. Technical specifications of the LFUS disinfection reactor (Vortex reactor WR 4-1402.03, Bandelin electronic, Berlin, Germany).

Technical Data	Vortex reactorWB 4-1402.3
Filling volume	~ 5 L
Ultrasound volume	2.9 L
Ultrasound distance	500 mm
Flow-throughh rate	60 - 3600 L/h
Reaction gap	15 mm
Power density max.	480 W/L
Frequency	25 kHz

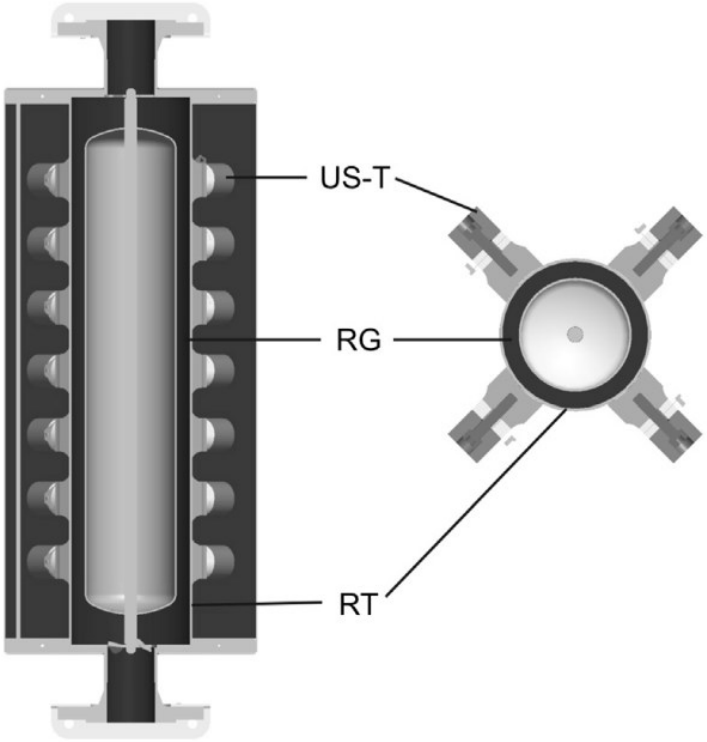

Figure 6. Assembly of the ultrasound disinfection reactor (Vortex reactor WR 4-1402.03, Bandelin electronic, Berlin, Germany), longitudinal section (**left**) and cross-section (**right**). **US-T**, transducers fixed at the reactor pipe; **RG**, narrow reaction gap; **RT**, reactor tube.

3.1.2 UV-C disinfection reactor

Due to the higher germicidal efficiency, smaller power draw and longer lamp life of LP-Lamps compared to the MP-Lamps, we used an UV-C disinfection reactor equipped with a LP-Lamp available on the market whose technical specifications (Micro light Basic 5; a.c.k. aqua concept, Karlsruhe, Germany) are summarized in Table 2.

Table 2. Technical specifications of the UV-C disinfection reactor (Micro light Basic 5; a.c.k. aqua concept, Karlsruhe, Germany).

Technical Data	Microlight Basic 5
Filling volume	8 L
Flow rate max.	3000 L/h
Electric power	110 W [a]
Efficiency of UV-lamp	31.8 %
UV-C power	35 W
UV-C dose at SAC_{254} = 22.18 1/m	min. 40 mJ/cm² [b]
UV-C dose at SAC_{254} = 70 1/m	min. 7.3 mJ/cm² [c]

[a] lamp without electronic ballast
[b] at 3000 L/h; ~ T_{10mm} = 60 % at the end of lamp-life
[c] personal communication with Dr. Gustav Cisk (a.c.k. aqua concept, Karlsruhe, Germany)

Both disinfection reactors were installed in a flow-through system equipped with a flow meter (300 to 3000 L/h) in which water first passed through the LFUS disinfection reactor followed by the UV-C treatment (Fig. 7). The volume-specific energy applied was adjusted by the variable power for LFUS disinfection reactor and changing of the retention time via the flow rate for LFUS and UV-C disinfection reactors. To compare the efficiency of LFUS and UV-C irradiation, dose-dependent reduction rates for model organisms were measured related to the volume-specific energy consumption and expressed as consumed specific energy. The consumed specific energy was measured for both disinfection reactors by deviation of power (W) to flow rate (L/s) and presented as kJ/L.

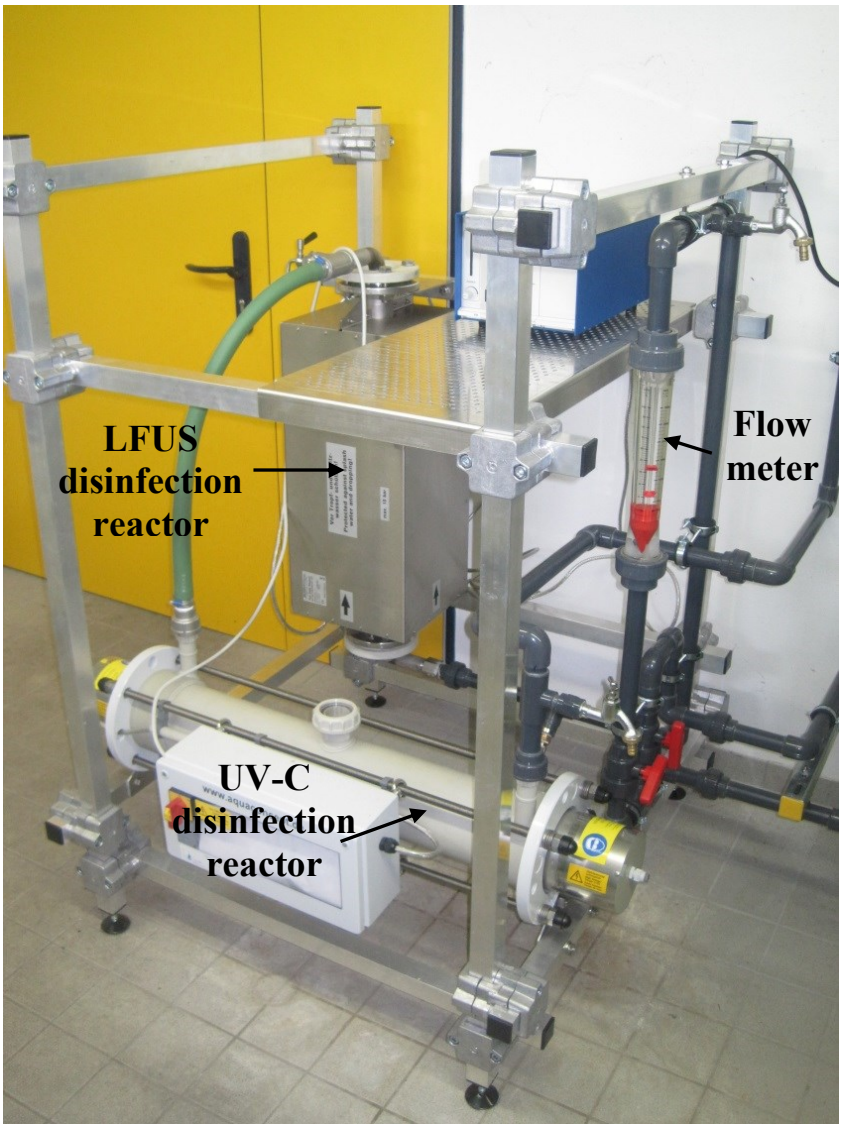

Figure 7. Flow-through LFUS and UV-C disinfection reactors.

3.1.3 Combined LFUS/UV-C disinfection reactor

After sole and combined application of LFUS and UV-C in a single-pass mode against model organisms, the combined LFUS/UV-C disinfection reactor was designed and constructed by Bandelin electronic, Berlin, Germany. The principle of this innovative disinfection reactor is the installation of a LP-lamp in the central tube of LFUS disinfection reactor (Fig. 8) for providing a combined treatment of the water by LFUS and UV-C in a single disinfection reactor. This idea was beneficial for saving space in RAS, as well as allowing access to a novel disinfection reactor system that can easily be installed in RAS.

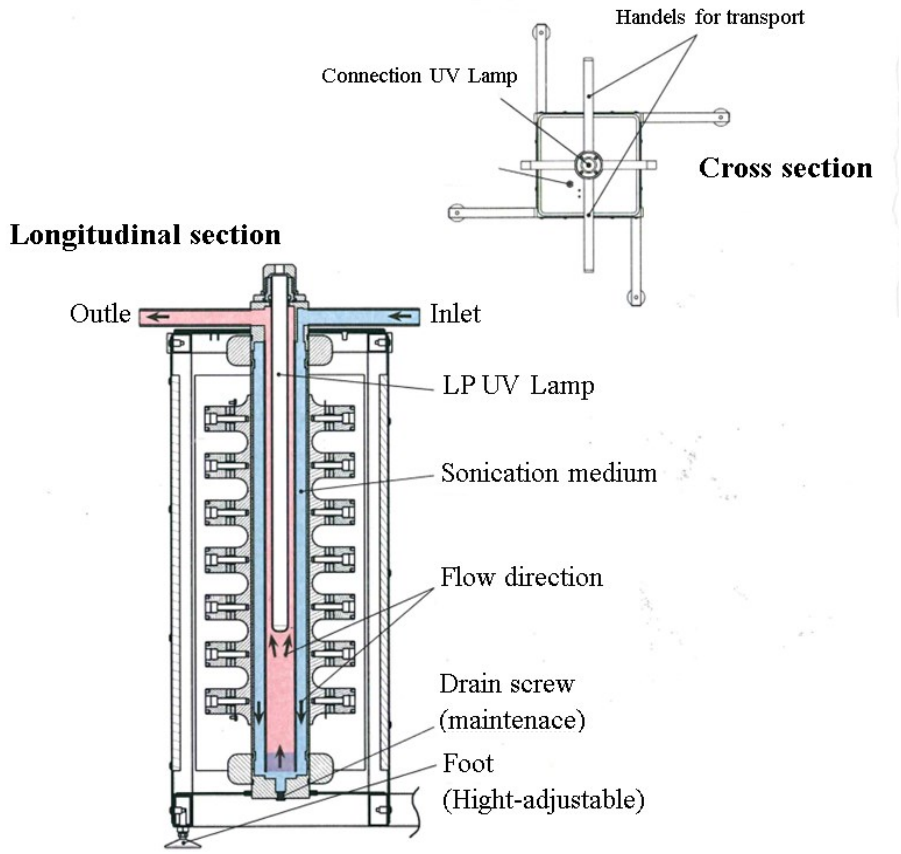

Figure 8. The longitudinal and cross section of combined LFUS/UV-C disinfection reactor. (Bandelin electronic, Berlin, Germany).

3.2 Source water

The source water used for the reduction experiment of prokaryotic and eukaryotic model organisms came from two RAS with different spectral attenuation coefficients at 254 nm (SAC_{254}). The first RAS system with SAC_{254} ranging from 24 to 35 1/m (27 ± 4 1/m) served as the representative of low UV-C attenuation systems and the second RAS with SAC_{254} ranging from 68 to 73 1/m (71 ± 2 1/m) served as the representative of high UV-C attenuation systems. SAC_{254} is the essential parameter for the design of UV disinfection reactor and accounts for the absorption and scattering of UV-C light by DOM and TSS, respectively. The determination of SAC_{254} was performed photometrically (Shimadzu UV/Vis-2401, Kyoto, Japan) in a 1 cm quartz cuvette and presented as 1/m. The absorbance spectrum between 200 - 400 nm including UV-C absorbance at 254 nm (Fig. 9) between filtered (Whatman 25-mm GD/X Syringe Filters, Nylon; pore size, 0.45 µm non-sterile) and unfiltered water was very similar and the difference was negligible. Therefore having the total attenuation of UV-C light we measured the SAC_{254} for unfiltered RAS-derived water.

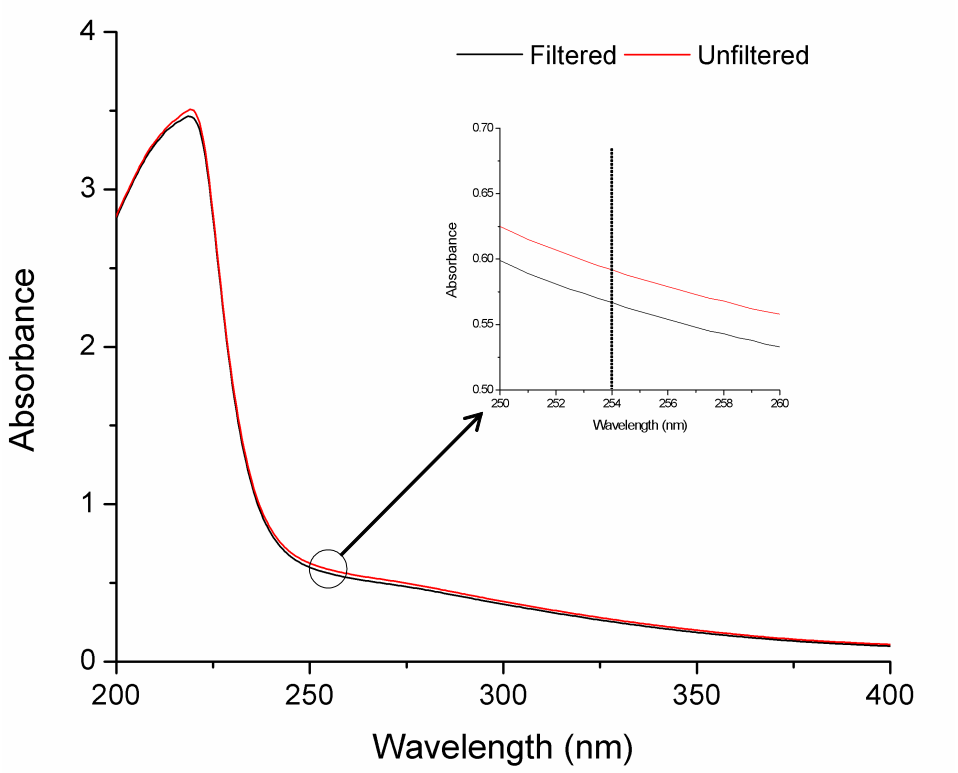

Figure 9. Absorption spectrum and UV-C (254 nm) absorbance of RAS-derived water.

3.3 Model organisms

Due to the prevalence of bacterial and parasitic disease in the aquaculture industry, the efficiency of LFUS and UV-C against prokaryotic and eukaryotic model organisms was examined. As models for taxa containing common fish pathogens in the aquaculture industry, the following organisms were chosen: heterotrophic bacteria naturally occurring in the water of RAS; the ciliate *Paramecium* sp.; second larval stage (L2) of the nematode *Anguillicola crassus* and metanauplii of *Artemia* sp. The model organisms which we used in this study covered the broad size range from small bacteria with a size of few micrometers to the larger *Artemia* sp. with a size of half a millimeter. The broad size range allowed us to evaluate the size-dependent reduction of model organisms by application of LFUS and UV-C treatment.

3.3.1 Bacteria

The total viable count (CFU/mL) was quantified by the spread plate technique using nutrient agar (DEV) (Carl Roth, Germany). Before inoculation, all water samples (50 ml) were dispersed for 10 s by sonication (20 kHz, 70 W; Sonopuls HD 7020,

Bandelin, Germany). In order to determine the bacterial count, the inoculated plates were incubated for 48 hours at 25 °C. Then, the total viable count was calculated from the number of visible colonies at the surface of the agar plate as CFU/mL (Fig. 10).

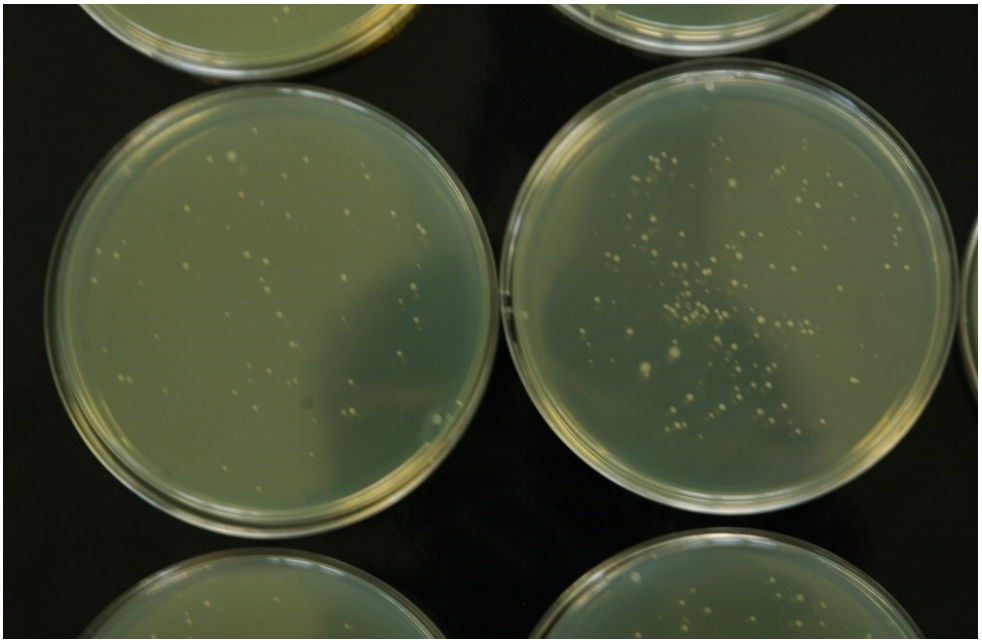

Figure 10. Colony forming units at the surface of agar plate.

Total bacterial count of water was also determined by using DAPI staining method (Fig. 11). Water samples were fixed with PBS buffer formaldehyde (the final concentration of formaldehyde in sample was 2 %) and transferred to the laboratory and stored at 4 °C until ready to count. The water samples were fixed with PBS buffer formaldehyde and incubated for 20 min with 4', 6-diamidino-2-phenylindole (DAPI) (Karl Roth, Germany) with the final concentration of 20 µg/mL. The water samples were filtered through a sterile 0.22 µm black polycarbonate membrane filter (Karl Roth, Germany). The filter paper containing the total bacteria was placed on a microscope slide and covered with Mounting Media (VECTASHIELD, VECTOR LABORATORIES, INC. USA) and covered with coverslip. The bacterial counting was performed by using fluorescence microscopy method and the total bacterial count was determined by using the following formula (Wetzel and Likens 1991):

Bacteria / ml= membrane conversion factor × ND

Membrane conversion factor = Filtration area / area of micrometer field

N = Total number of bacteria counted / number of micrometer fields counted

D = Dilution factor

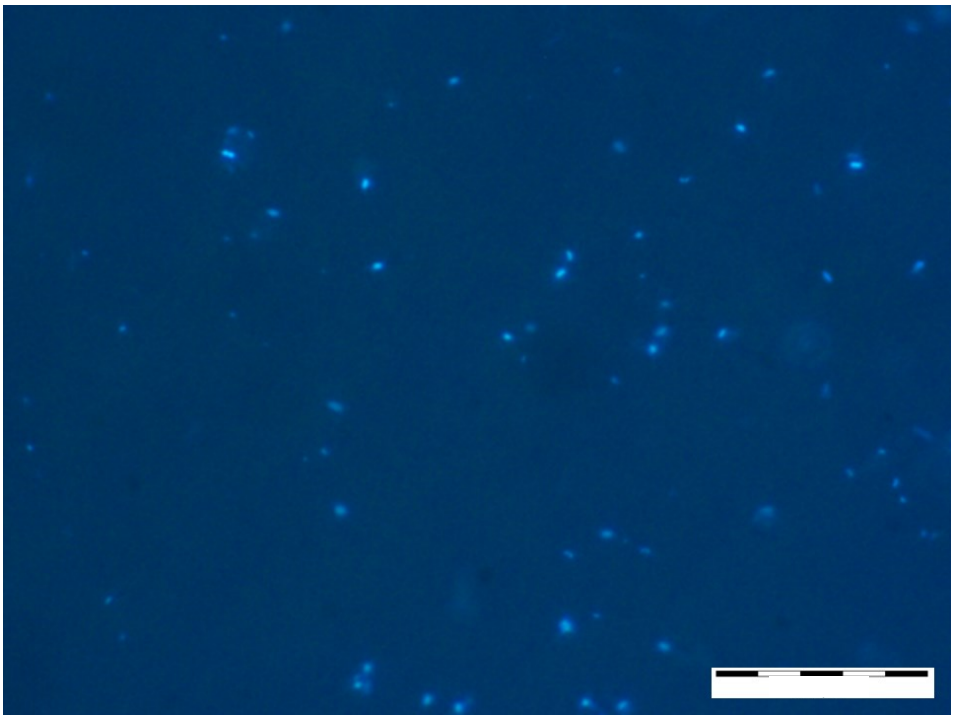

Figure 11. Bacteria stained with DAPI, representing the total bacterial count of the water sample. Scale bar: 20 µm.

3.3.2 Eukaryotic organisms

To test the efficiency of LFUS and UV-C against eukaryotic parasites, three groups of eukaryotic model organisms representing the most important taxa of parasitic organisms in the aquaculture industry and also free swimming *Trichodina* sp. obtained from a RAS were chosen. The first model organism in this study was *Artemia* sp. as model organism for crustacean. It was obtained from hatching dried cysts (INVE aquaculture, Belgium). We used the metanauplii of *Artemia* sp. three days post hatch (3 DPH) with a size ranging from 500 - 700 µm.

The second model organism was *Paramecium* sp. as model for ciliated ectoparasites. We used the *Paramecium* sp. from hay infusion after 4 days with the size ranging from 70 - 110 µm. Second stage larvae (L2) of *Anguillicola crassus* were collected from the swim bladders of naturally infected European eels *Anguilla anguilla* from the

Müggelsee, Berlin, Germany as the representative of parasitic nematodes. The length of these nemathode larvae ranged from 250 - 320 µm.

After preparation of the eukaryotic model organisms, the approximate total number of animals in the stock tank was determined. The source water was spiked with model organisms just before starting the experiment and thoroughly mixed by aeration for achieving a homogenous distribution of model organisms in the water.

Water samples containing *Paramecium sp.* were fixed with Lugol's iodine, 3 ml per well of 24-well microtiter plates and allowed to settle for 60 min and were counted using an inverted microscope. Individuals with an abnormal, spherical shape were considered to be irreversibly harmed. Non-fixed samples containing L2 of *Anguillicola crassus* were allowed to settle in 50 ml Utermöhl chambers (HYDRO-BIOS Apparatebau GmbH, Germany) for 30 min and were counted by using an inverted microscope. Immotile nematodes not responding to a physical stimulus applied with a fine needle were considered to be dead. Viable *Artemia* sp. metanauplii were counted using a zooplankton counting chamber (HYDRO-BIOS Apparatebau GmbH) under binocular.

3.4 Experiments

3.4.1 Effect of LFUS on particle size distribution

Due to the shielding role of TSS for embedded organisms, mainly bacteria, (Simon et al. 2002), the efficiency of disinfection methods can be affected by the presence of suspended solids. The size distribution of TSS can easily be determined by Laser Diffractometry (LD). For a quick measurement of the geometrical dimensions of TSS, laser diffraction analysis utilizes patterns of a laser beam passed through any medium such as water which contains particles ranging from nanometers to millimeters in size (Stojanovic and Marcovic 2012). This method will give a volume-weighted distribution, meaning the contribution of each particle in the distribution related to the volume of that particle. The most common percentiles reported are d (0.1), d (0.5) and d (0.9) which are also recognized as the median particle size by volume. For example, d (0.1) = 21 µm is the particle diameter which 10 % is smaller than 21 µm and 90 % is bigger than 21 µm. The aim of this study was to show the efficiency of LFUS to reduce the particle size, a process favorable for conventional disinfection reactor such as UV-C (Blume and Neis, 2004). The water of a RAS stocked with Nile tilapia *Oreochromis niloticus* with a

stocking density of 60 Kg/m^3 and TSS of 8.5 ± 0.8 mg/L was exposed to different consumed specific energies of 1.9, 3.8 and 19 kJ/L. The amount of TSS was measured according to standard methods procedure 2540 D (Clesceri 1998). The particle size distribution of the water treated with different LFUS energies was measured by Laser Diffractometry (LD) (Mastersizer 2000, Malvern, UK) within 2 hours post sampling.

3.4.2 Mathematical model

The least LFUS and UV-C intensity required for reduction of model prokaryotic and eukaryotic organisms in a continuous-pass mode was estimated with a mathematical model assuming exponential growth and a linear reduction of organism in circulating water:

$$N_t = N_0 \times e^{\left(\ln\left(\frac{2}{t_g}\right) - p \times \frac{Q}{V}\right)} \times t$$

with

N_t = number of organism at time t

N_0 = initial number of organism

t_g = generation time of the organism

p = reduction rate

Q = flow rate

V = total volume of the system

t = time

3.4.3 Reduction of bacteria

The efficiency of sole and combined application of LFUS and UV-C against bacteria was evaluated in two steps: single-pass mode of RAS-derived water and continuous-pass mode in a RAS. The reduction rate of heterotrophic bacteria in a dose dependent mode was evaluated for both steps by using the total viable count (CFU/mL) and total bacterial count by DAPI staining method. The reduction rate was calculated as the ratio between bacterial counts after and before treatment and the lower counts in the treated samples were considered to be reduced due to the treatment

3.4.3.1 Single-pass mode

In single-pass mode, the application of LFUS against bacteria in a flow–through system was examined both individually and as a pretreatment before UV-C irradiation. Both LFUS and UV-C disinfection reactors were installed in a pilot flow-through system. The range of consumed specific energies of LFUS and UV-C were achieved by adjusting the flow rate (3000 - 300 L/h) was 1.9 - 19 kJ/L and 0.13 - 1.3 kJ/L, respectively. The water samples were immediately transferred on ice to laboratory for bacterial counting.

3.4.3.2 Continuous-pass mode

Studies on the efficiency of an UV-C disinfection reactor operating in a bypass mode were performed in two RAS with a total water volume of 12 m^3, each, stocked with Nile tilapia, *Oreochromis niloticous* with a stocking density of 12.5 kg/m^3. In one system, a 110 W UV-C disinfection reactor was operated in full-flow mode (133 % of RAS water volume per hour) or in a bypass mode (67 % of RAS water volume per hour). Finally, a 110 W UV-C disinfection reactor was operated following a pretreatment with LFUS (1.4 kW) in a bypass mode (25 % of RAS water volume per hour). The second RAS, not treated with UV-C, was used as control. The duration of all experiments was 96 h, and each day the total viable count (CFU/mL) was determined by the spread-plate technique using a nutrient agar (DEV) (Carl Roth, Germany). Additionally, the total bacterial count was determined by DAPI staining method by means of a fluorescence microscope. During the experimental period, the bacterial count in each day was compared to initial bacterial count (0 h) and expressed as relative count.

3.4.4 Reduction of eukaryotic organisms

For the single-pass mode, the effects of sole applications of UV-C and LFUS against *Artemia* sp., *Paramecium* sp. and *Anguillicola crassus* as the eukaryotic model organisms were examined. A comparison of sole applications of LFUS and the combined effect of LFUS/UV-C was evaluated for the *Artemia* sp. In addition to the eukaryotic model organisms, the application of LFUS against free-swimming *Trichodina* sp. as a real ciliated ectoparasites was also evaluated in single and continuous-pass modes. The dose dependent reduction rate of eukaryotic model organisms and *Trichodina* sp. was determined by counting by means of microscopy

methods. The reduction rate was calculated as the ratio between viable organisms after and before treatment and the lower counts in the treated samples were considered to be reduced due to the treatment

3.4.4.1 Single-pass mode

In single-pass mode the RAS-derived water with SAC_{254} of 27 ± 4 1/m was spiked with the eukaryotic model organisms. The consumed specific energies of LFUS and UV-C ranged from 1.9 - 19 kJ/L and 0.13 - 1.3 kJ/L, respectively. In these experiments we evaluated the sole and combined effect of LFUS and UV-C disinfection reactors in a single-pass mode. In combination mode, water was passed first through the LFUS disinfection reactor and then introduced to the UV-C disinfection reactor. The effect of LFUS at constant specific consumed energy (1.9 kJ/L) but variable power in different SAC_{254} (27 ± 4 1/m and 71 ± 2 1/m) on the reduction of *Artemia* sp. metanauplii were also examined.

The dose-dependent reduction rate of free-swimming *Trichodina* sp. obtained from a RAS with a total volume of 16 m^3, stocked with European sturgeon *Acipenser sturio* with a stocking density of 6.2 kg/m^3, was also determined. Clinical symptoms of the fishes such as jumping, gathering at the water inflow and rapid gasping indicated an infection with ectoparasites. Parasitological examination confirmed the infection of the fish with *Trichodina* sp. with a size range of 50 - 100 µm. For this reason the consumed specific energies of 0, 0.8, 1.9, 6 and 19 kJ/L were applied to evaluate the reduction rate of *Trichodina* sp. after a single-pass through the LFUS disinfection reactor. In this study we measured the number of free-swimming *Trichodina* sp. and did not measure the number of *Trichodina* sp. attached to the skin. The water contained the eukaryotic organisms was sampled after each treatment and immediately transferred to the laboratory for microscopic counting.

3.4.4.2 Continuous-pass mode

After evaluating the reduction of free-swimming *Trichodina* sp by LFUS in a single-pass mode, the efficiency of LFUS against free-swimming *Trichodina* sp was examined in a continuous-pass mode up to 96 h. The mathematical model for *Trichodina* sp. allows us to estimate the number of free-swimming *Trichodina* sp. after a certain treatment with a certain dose. Consequently, two LFUS disinfection reactors, each of

them operated at 1.4 kW (25 kHz) and 3 m^3/h, were installed in parallel to sonicate 38 % of RAS water volume per hour. The duration of this experiment was 96 h and the water was sampled daily to check the reduction rate of free-swimming *Trichodina* sp. Water samples were fixed with Lugol's iodine and allowed to settle for 24 h in 50 ml Utermöhl chambers (HYDRO-BIOS Apparatebau GmbH, Germany). *Trichodina* sp. with an abnormal and broken shape were considered to be irreversibly harmed and dead by LFUS and was counted by using an inverted microscope (Fig. 12).

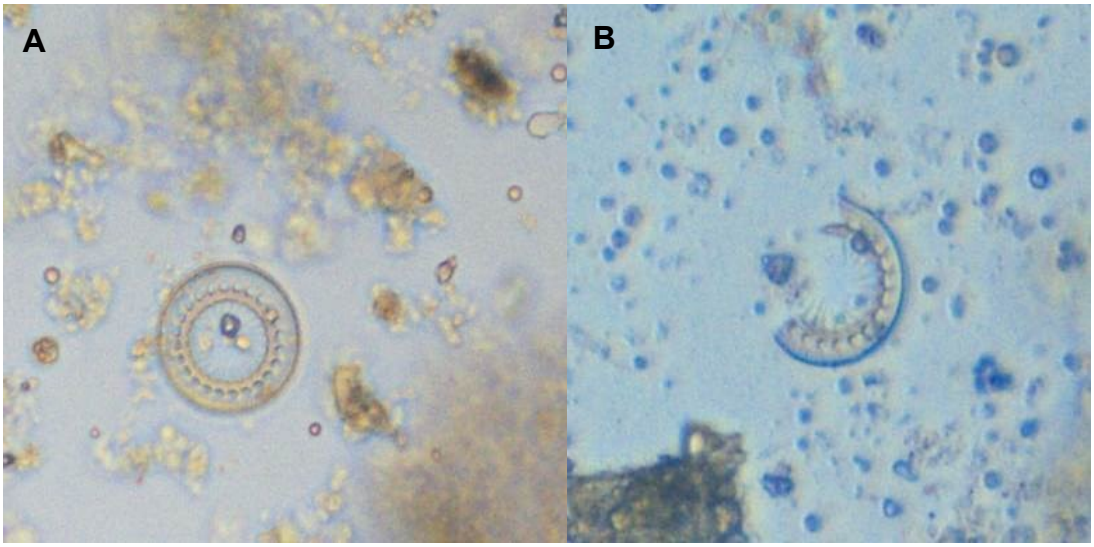

Figure 12. Visually non-affected (**A**) and harmed (**B**) *Trichodina* sp. by LFUS in RAS–derived water.

The mathematical model can be used for calculating the required power at a given flow rate, volume, and expected reduction. For t_g = 24 h for *Trichodina* sp. (Feng, 1985), V = 16 m^3 and Q = 6 m^3/h, the model reveals that a reduction rate of 25 % by LFUS could result in a reduction rate of free-swimming *Trichodina* sp. by 90 % within 2 days.

3.4.5 Photoinduced formation of NO_2^- from NO_3^-

Photoinduced formation of NO_2^- from NO_3^- was evaluated by using a flow-through disinfection reactor equipped with a LP lamp (Micro light Basic 5; a.c.k. aqua concept, Karlsruhe, Germany). Technical specifications of the UV-C disinfection reactor are summarized in Table 1. Since the effective UV-C dose strongly depends on SAC_{254} of the treated water, the applied UV-C doses are expressed as the volume-specific UV-C energy input, where 0.042 kJ/L corresponds to 40 mJ/cm^2 for SAC_{254} = 22.18 1/m. The applied UV-C doses ranged from 0.042 to 6.3 kJ/L after adjustment to the retention time

of the flow-through disinfection reactor. Experimental water with different NO_3^- concentrations (3, 100, 300, 800 and 1200 mg/L) was prepared by adding sodium nitrate, $NaNO_3$ (Karl Roth, Germany) to the tap water with 3 mg/L NO_3^- and < 0.03 mg/L NO_2^-. All treatments were carried out at 13 °C and 27 °C with 8 replicates, and the pH value remained 7.6 throughout. Water samples were taken from the ultraviolet disinfection reactor outlet and the NO_2^- level was immediately determined by using the Griess' reaction according to DIN EN 26777 (1993). The transformation rate of NO_3^- to NO_2^- was calculated as the percentage of the initial NO_3^--N that was transferred to NO_2^--N. For each NO_3^- concentration, the absorbance and transmittance spectrum from 200 nm to 400 nm was determined photometrically (Shimadzu UV/Vis-2401, Kyoto, Japan) in a 1 cm quartz cuvette. Additionally, according to the estimated gap width of the UV disinfection reactor used in this study, the transmittance was also determined with a 5 cm cuvette. Ultrapure water (Milli-Q, Millipore, Germany) was used as negative control.

3.4.6 Ecotoxicological tests

Evaluation of whole effluent toxicity (WET) for possible formation of toxic DBPs were evaluated by two ecotoxicological tests including fish egg test (FET) and luminescent bacteria test. Zebrafish egg test is the most promising alternative approach to classical acute fish toxicity (Lammer et al. 2009). Luminescent bacteria test is a common method in waste water analyses for testing the toxicity of unknown samples and partly stipulated in ordinances (e.g. German Wastewater Ordinance) (Escher et al. 2008). Zebra fish *Danio rario* eggs and luminescent bacteria *Vibrio fischeri* were exposed to water sonicated with the consumed specific energies of 19 kJ/L, water irradiated with UV-C with consumed specific energy of 1.3 and 6.3 kJ/L and combination of LFUS 19 kJ/L and UV-C 6.3 kJ/L. The water samples were transferred to the laboratory and the tests were performed within 3 hours after sampling.

3.4.6.1 Fish egg test (FET)

A fish egg test was performed according to DIN 38415-T6 (2001). Briefly, the fertilized eggs in an 8-cell stage were identified under a binocular microscope and selected for the test. Following suspension in the treated water or any of the controls, 10 fertilized eggs were selected and transferred to 24-well plates filled with 2 mL treated

water and controls per well. 3,4-Dichloroaniline (3,4-DCA) was used as positive control and dilution water was used as negative control. The plates were then covered and incubated at 26.0 ± 1.0 °C. Four lethal toxicological endpoints including coagulation of the eggs, failure to develop somites, lack of heart-beat and non-detachment of the tail from the yolk were recorded after 24 h and 48 h.

3.4.6.2 Luminescent bacteria test

The luminescent bacteria test is based on the inhibition of the enzyme luciferase, which oxidizes luciferin to generate light. Positive results indicate that a substance interferes with the energy metabolism of the cell and negatively affects the vitality and light emission of test luminescent bacteria *Vibrio fischerei*. The luminometry has a very low detection limit for toxic substances and allows for precise and quantitative measurements of the cell vitality of these bacteria. The luminescent bacteria test by using the bacterial strain DSM 7151 was performed according to DIN EN ISO 11348-2 (1998). Reconstituted luminescent bacteria were exposed to the test water (adjusted to the salinity of 20 g/L with NaCl) and reference substances (19.34 mg/L Zinc sulfate heptahydrate, 105.8 mg/L Potassium dichromate and 6.8 mg/L 3,5-Dichlorphenol). The bioluminescence properties of the bacteria were measured after an exposure time of 30 min by using a luminometer (LUMI Stox 300, Dr. Bruno Lange GmbH & Co KG, Düsseldorf, Germany).

3.4.7 Statistical analysis

Results were presented as the mean ± SD of n replicates. Data were analyzed for normal distribution by Kolmogorov–Smirnov and Kruskal-Wallis test for equal variance. For bacterial reduction, the non-parametric Kruskal–Wallis test, followed by a Dunn's multiple comparison test was used to determine statistically significant differences for CFU and DAPI values for single and continuous-pass modes. In single-pass mode, the effects of SAC_{254} and LFUS pretreatment on the germicidal effect of UV-C were evaluated using the Mann–Whitney U-test. For eukaryotic model organisms, non-linear regression analysis of the dose-dependent reduction rates by LFUS were performed using Origin Pro 8 SR 2 (OriginLab, Northampto n, MA). Mean values of the reduction rates for *Artemia* sp. were compared between different treatments using an analysis of variance (ANOVA), followed by Bonferroni post hoc test. Size percentiles of the

suspended solids in sonicated and non-sonicated samples were compared using an analysis of variance (ANOVA), followed by Bonferroni post hoc test. Spearmann rank correlation was used to test the relationship between photoinduced NO_2^- formation with NO_3^- concentration and UV-C dose, as well as the transformation rate with nitrate concentration and UV-C dose. If not mentioned differently, all statistical analyses were performed by using Graph Pad Prism 4.03 (Graph Pad Software, Inc., San Diego, CA).

4 Results

4.1 Effect of LFUS on particle size distribution

The results of particle size distribution study showed that the volume weighted distribution of suspended particles in RAS-derived water had a significant tendency towards smaller particle sizes when LFUS was applied with maximum consumed specific energy of 19 kJ/L compared to 1.9 and 3.8 kJ/L and control group (Fig.13).

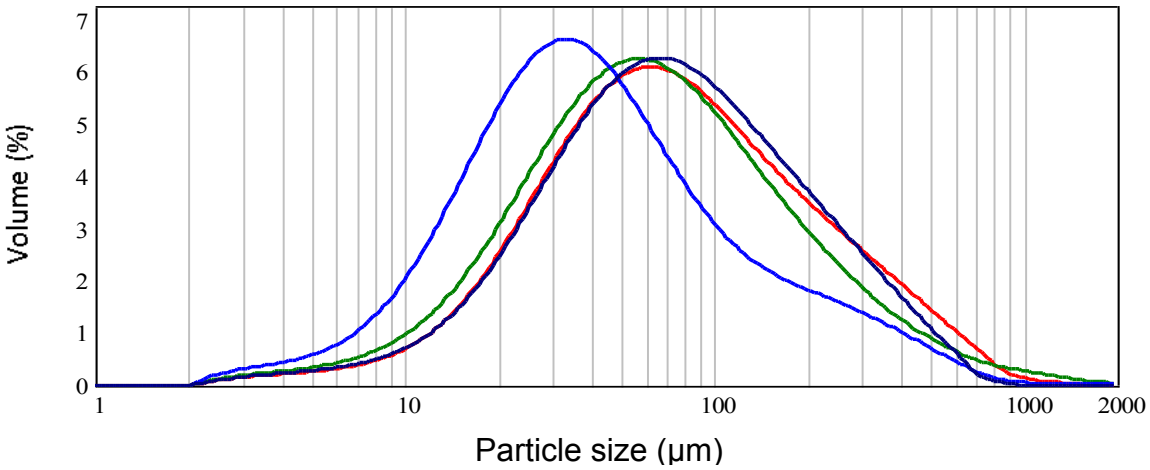

Figure 13. Effect of LFUS applied with consumed specific energies of 0 kJ/L (**Red**), 1.9 kJ/L (**dark blue**), 3.8 kJ/L (**green**) and 19 kJ/L (**blue**) on frequency of particle size distribution in RAS-derived water.

Analysis of percentiles also showed a significant particle size reduction ($p < 0.05$) in a dose dependent manner. The strongest effect was observed when water was exposed to the maximum consumed specific energy of 19 kJ/L (Fig. 14). The percentiles d (0.1) = 21.1, d (0.5) = 72.8 and d (0.9) = 262.7 µm before sonication changed to d (0.1) = 12.3, d (0.5) = 38.8 and d (0.9) = 187.6 µm after sonication which showed 42, 33 and 26 % particle size reduction, respectively.

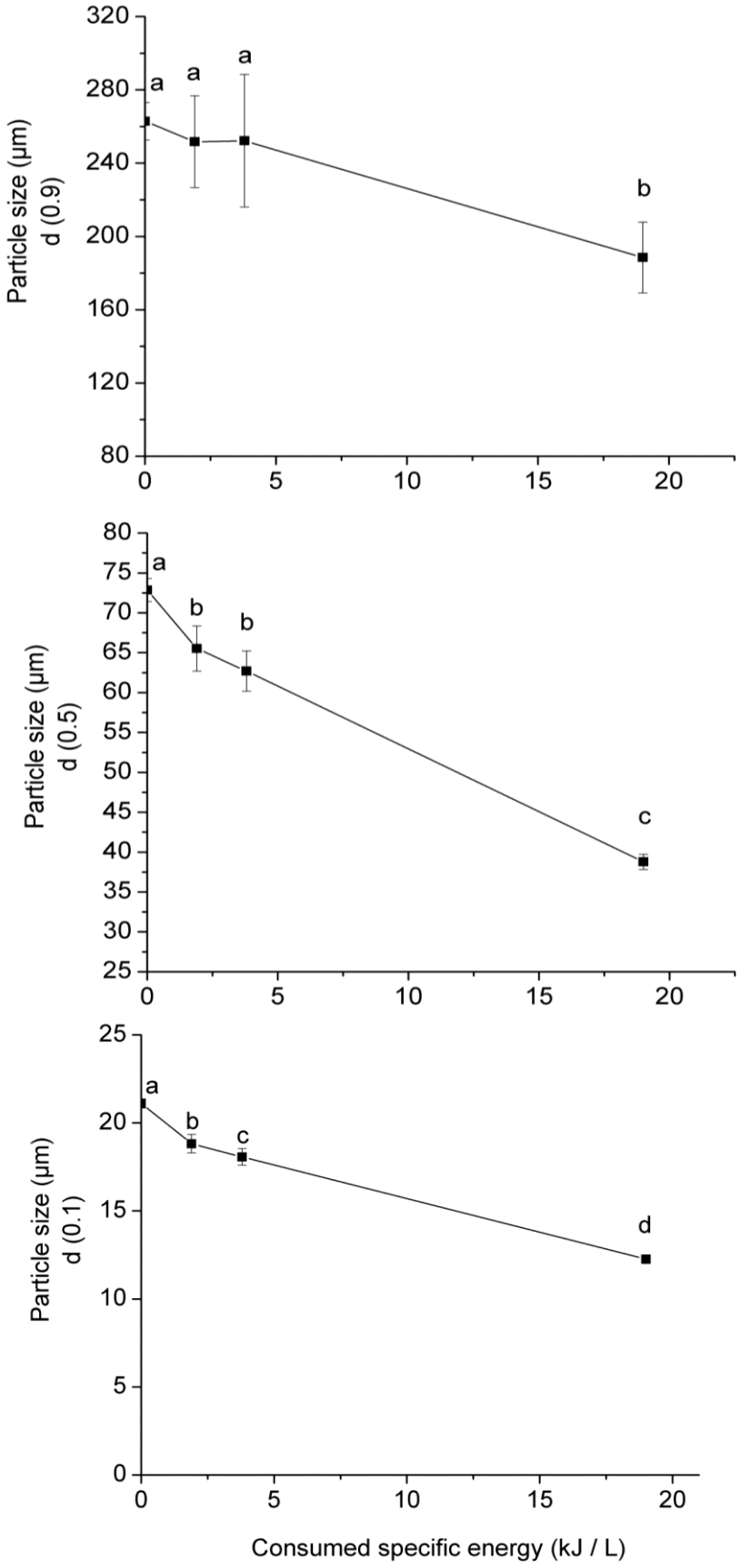

Figure 14. Effect of LFUS on the percentiles of particle size. Data are presented as mean ± SD (n = 6), different superscripts denote significant differences between groups ($p < 0.05$).

4.2 Reduction of bacteria

4.2.1 Single-pass mode

The single application of LFUS with consumed specific energy of 1.9 - 19 kJ/L (3000 - 300 L/h) in water with low and high SAC_{254}, did not result in a significant reduction of total viable count (CFU/mL) compared to the control group (Fig. 15A and B). UV-C irradiation with a consumed specific energy of 0.13 - 1.3 kJ/L (3000 - 300 L/h) resulted in a significant reduction of the total viable count compared to the control group ($p < 0.001$, Fig. 15A and 2B). In water with low SAC_{254} (SAC_{254} = 27 1/m), UV-C irradiation was significantly more effective than in water with high SAC_{254} (SAC_{254} = 711/m) ($p < 0.001$, Fig. 15A and B). Compared to the control group, the CFU values were reduced by 2.0 - 2.5 log units in water with low SAC_{254} (SAC_{254} = 27 1/m) ($p < 0.001$, Fig. 15A), but only by 1.1 - 1.7 log units in water with high SAC_{254} ($p < 0.001$, Fig. 16B). Compared to sole UV-C irradiation, pre-treatment with LFUS did not result in an additional bacterial reduction in water with low SAC_{254} (SAC_{254} =271/m) (Fig. 15A), but it increased the germicidal effect of UV-C by up to 0.6 log units in water with high SAC_{254} (SAC_{254} = 71 1/m) (Fig. 15B).

Determination of total bacterial count (bacteria/mL) by DAPI staining method showed no significant differences after the application of maximum LFUS energy (19 kJ/L) and even after the UV-C treatments(1.3 kJ/L) compared to the control group (Fig.16 and 17).

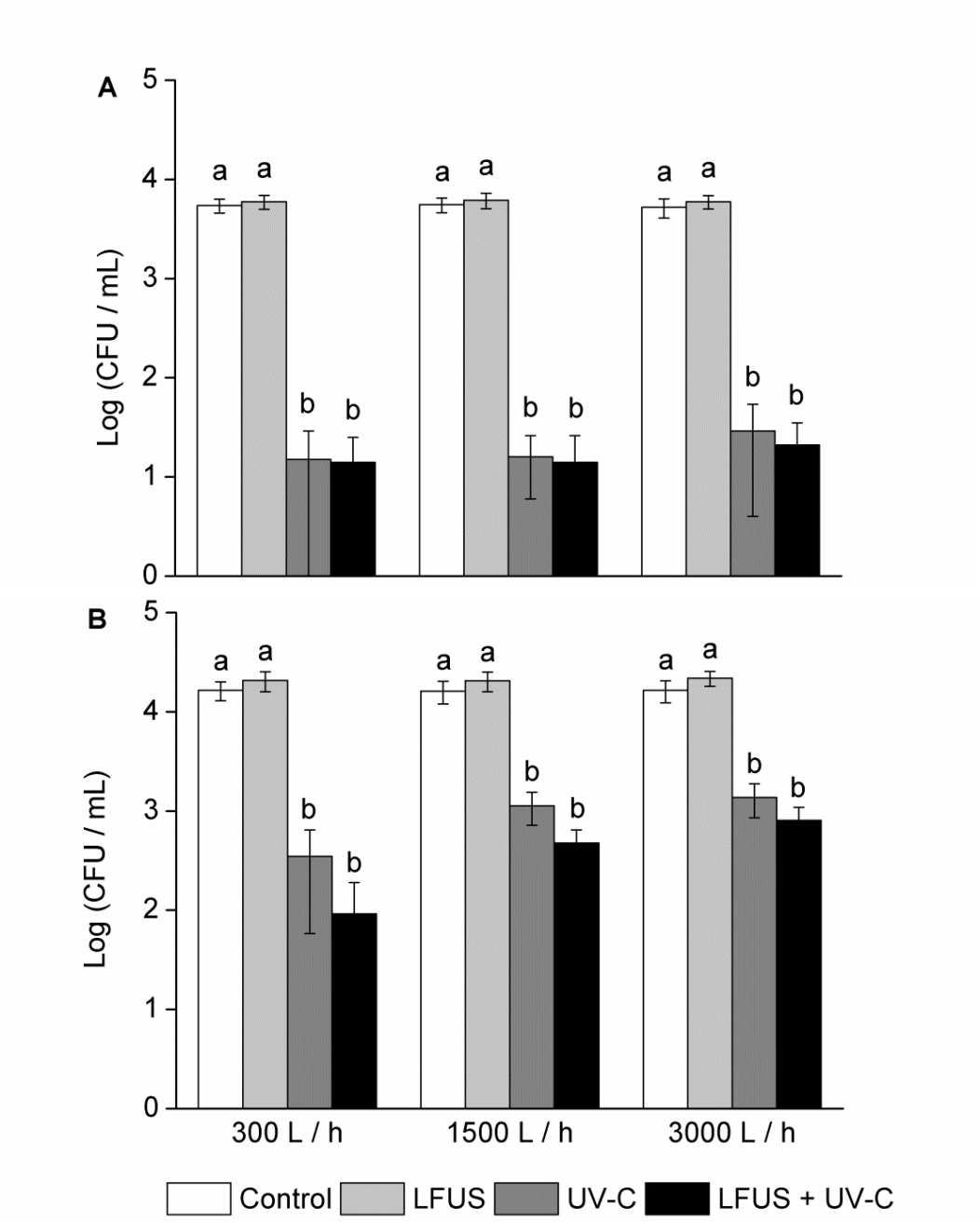

Figure 15. Effect of LFUS, UV-C and the combination of both treatments on the total viable count (CFU/mL) at different spectral attenuation coefficients of (**A**) SAC_{254} = 27 1/m and (**B**) SAC_{254} = 71 1/m. Data are presented as mean ± SD (n = 15), different superscripts denote significant differences between groups ($p < 0.05$).

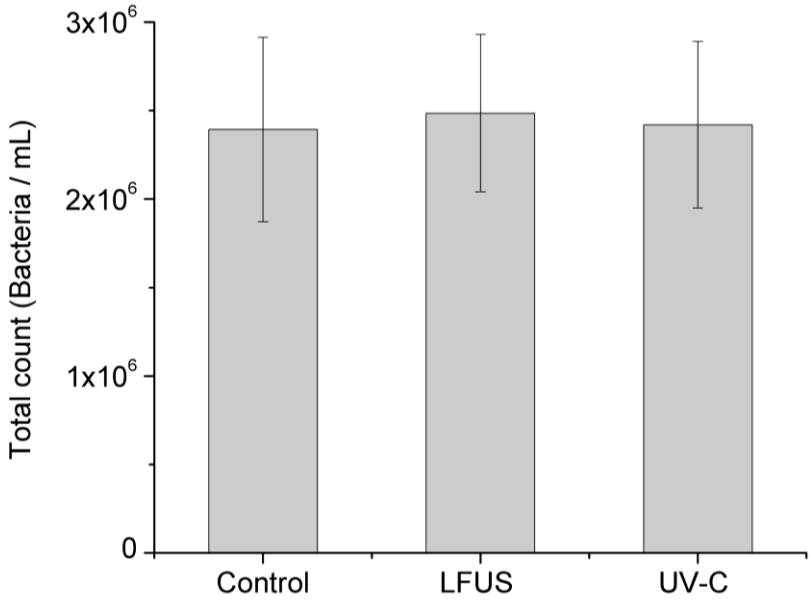

Figure 16. Effect of single LFUS (19 kJ/L) and UV-C (1.3 kJ/L) treatments on total bacterial count determined by DAPI staining method at spectral attenuation coefficients of SAC_{254} = 27 1/m. Data are presented as mean ± SD (n = 10). No significant differences observed between groups ($p > 0.05$).

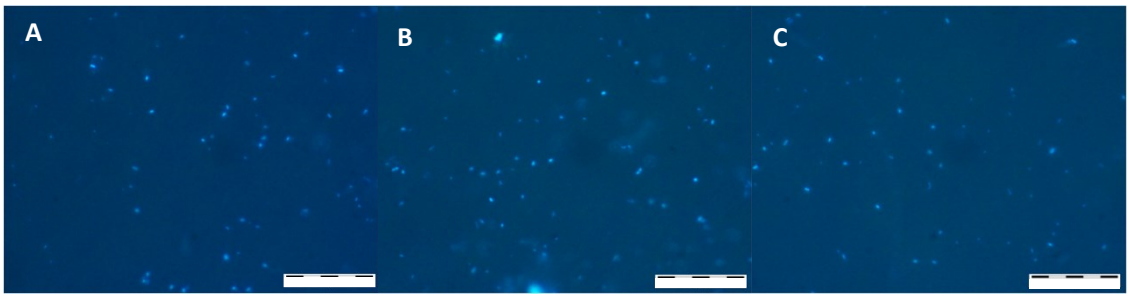

Figure 17. DAPI stained bacteria following single LFUS (19 kJ/L) (**B**) and UV-C (1.3 kJ/L) (**C**) treatment compared to the control group (**A**). Scale bar: 20 µm.

4.2.2 Continuous-pass mode

UV-C irradiation in an experimental RAS equipped with a 110 W UV-C disinfection reactor significantly reduced the amount of total viable count within 96 h when UV-C disinfection reactor was operated in a full-flow mode (133 % RAS water volume per hour). During the course of this study (96 h), the bacterial reduction rate determined by total viable count was 80 % (Fig. 18A), whilst evaluation of DAPI stained samples revealed a 90 % reduction in total bacterial count (Fig 18 B, 19 A and B).

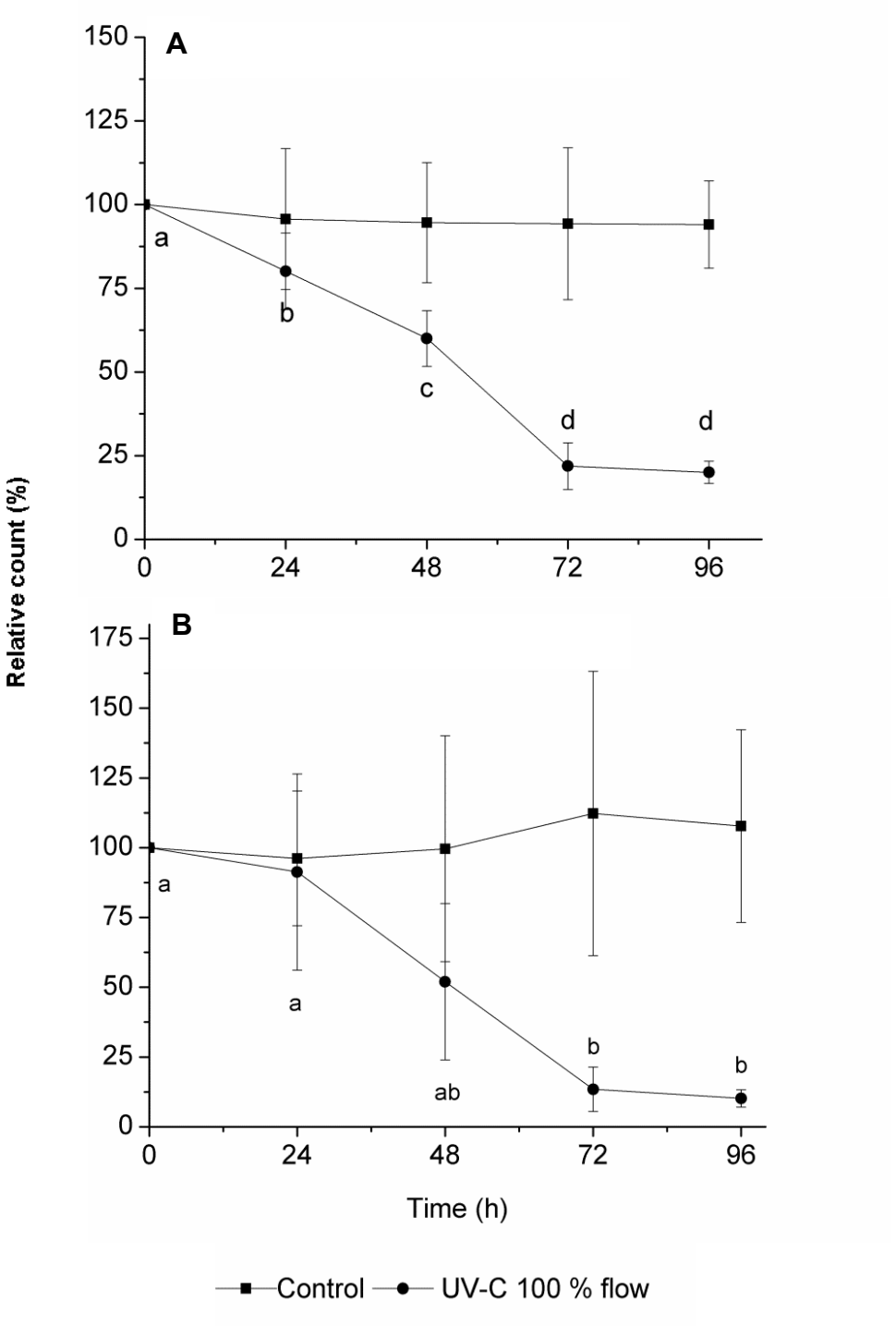

Figure 18. Effect of UV-C irradiation in a full-flow mode on total viable count (CFU/mL)(**A**) and total bacterial count determined by DAPI staining method (bacteria/mL)(**B**). Data are presented as mean ± SD (n = 15), different superscripts denote significant differences within UV-C treated group ($p < 0.05$).

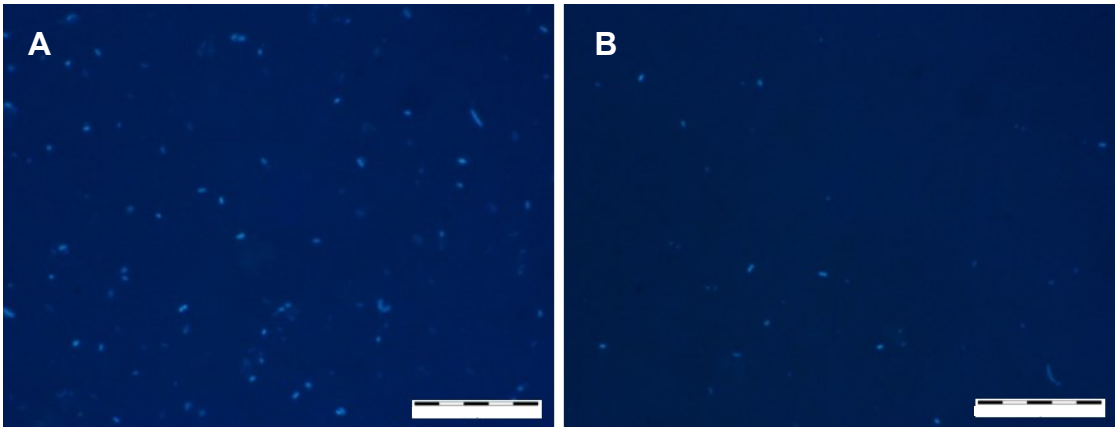

Figure 19. Effect of UV-C irradiation in a full-flow mode on total bacterial count in 0 h (**A**) and 96 h (**B**) post treatment determined by DAPI staining method. Scale bar: 20 µm.

In spite of the significant reduction of free viable heterotrophic bacteria in treated RAS with UV-C irradiation in a full-flow mode, the bacterial reduction proceeded much more slowly than the mathematically predicted reduction of bacterial count (Fig. 20).

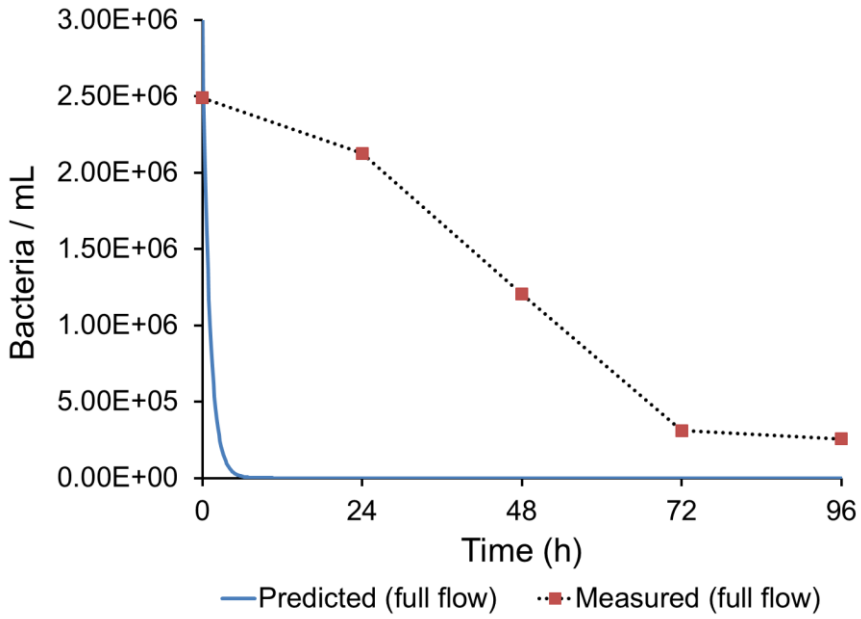

Figure 20. Theoretical and measured reduction of bacterial count (generation time of 2.7 h and reduction by only one log unit) in a RAS by full-flow application of UV-C.

The same UV-C dose had no effect on the total viable count when the UV-C disinfection reactor was operated in a bypass with 67 % of RAS water volume per hour for the same period (96 h) (Fig. 21A). Similarly, when a 110 W UV-C disinfection reactor was operated in a bypass with 25 % of RAS water volume per hour following a pretreatment with LFUS (1.4 kW), no significant difference in the total viable count was

observed between the treated and control RAS. However, the relative count revealed a significant reduction within the RAS treated by LFUS after 24 h which was significantly lower compared to the initial (0 h) and final (96 h) sampling time (Fig. 21 B).

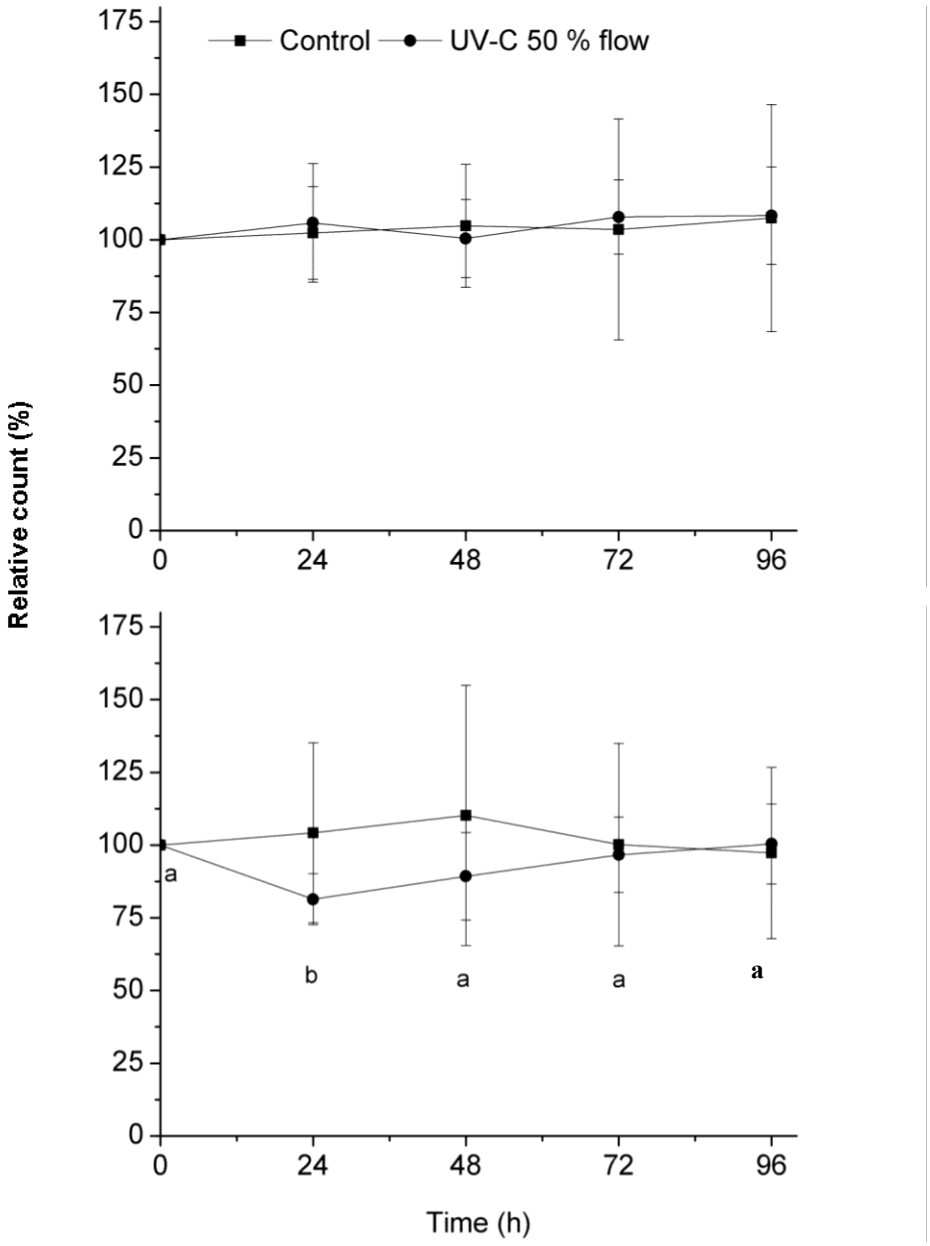

Figure 21. Effect of sole UV-C irradiation in bypass mode (67 % of RAS water volume per hour) (**A**) and combined with LFUS in bypass mode (25 % of system volume per hour) (**B**) on total viable count. Data are presented as mean ± SD (n = 9), different superscripts denote significant differences within LFUS treated group ($p < 0.05$).

4.3 Reduction of eukaryotic organisms

4.3.1 Single-pass mode

The application of UV-C doses of 0.13 - 1.3 kJ/L (consumed specific energy) against eukaryotic model organisms resulted in reduction rates ranging from 8 - 41 % for *Paramecium* sp., 37 - 76 % for *Anguillicola crassus* larvae and 8 - 82 % for *Artemia* sp. (Fig. 22).

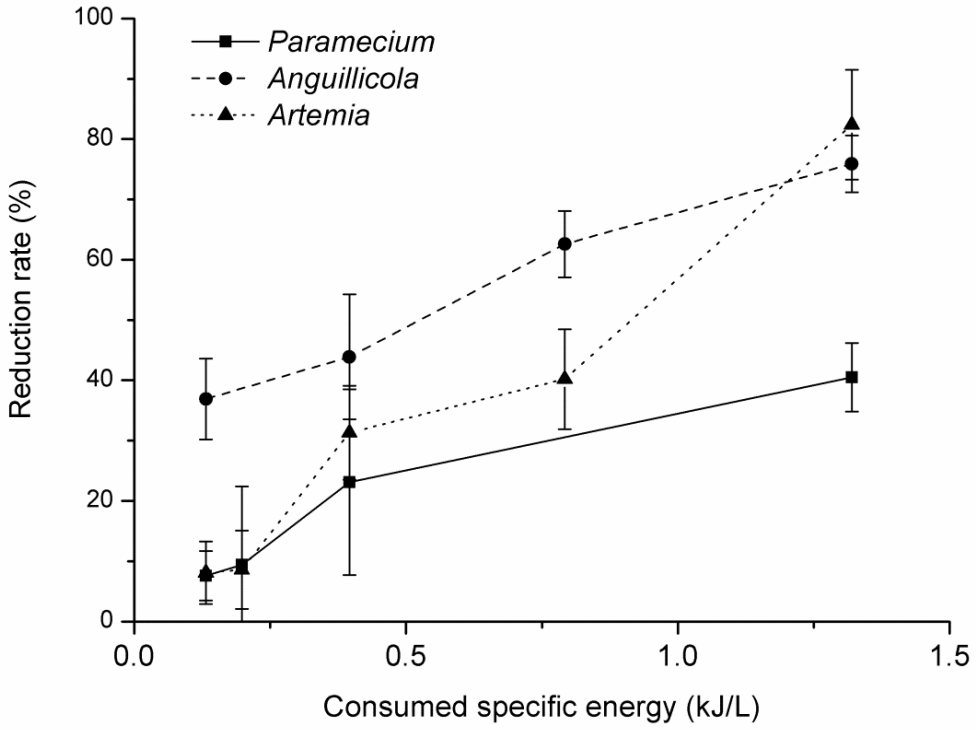

Figure 22. Dose-dependent reduction rate of *Paramecium* sp., second-stage larvae of the nematode *Anguillicola crassus* and metanauplii of *Artemia* sp. by UV-C irradiation. Data are presented as mean ± SD (n = 5).

A sole application of LFUS with 0.19 - 19 kJ/L resulted in dose-dependent reduction rates of *Paramecium* sp. ranging from 7 to 95 %. For *Anguillicola crassus* larvae, LFUS ranging from 1.9 to 19 kJ/L resulted in reduction rates ranging from 19 - 81 %, and LFUS doses ranging from 0.19 to 1.9 kJ/L resulted in 70 - 99 % reduction rates of *Artemia* sp. (Fig. 23). The dose-dependent reduction of eukaryotic model organisms can be described by functions of an exponential decay. In the interval 0 - 19 kJ/L the best-fit decay functions were:

Paramecium sp.: $Y = 104 - 103 \exp(-X / 7.30)$; $r^2 = 0.990$

Anguillicola crassus: $Y = 83 - 79 \exp(-X / 5.19)$; $r^2 = 0.979$

Artemia sp.: $Y = 100 - 101 \exp(-X / 0.34)$; $r^2 = 0.998$

where Y is reduction rate in % (defined for $0 \leq Y \leq 100$) and X is consumed energy in J/L.

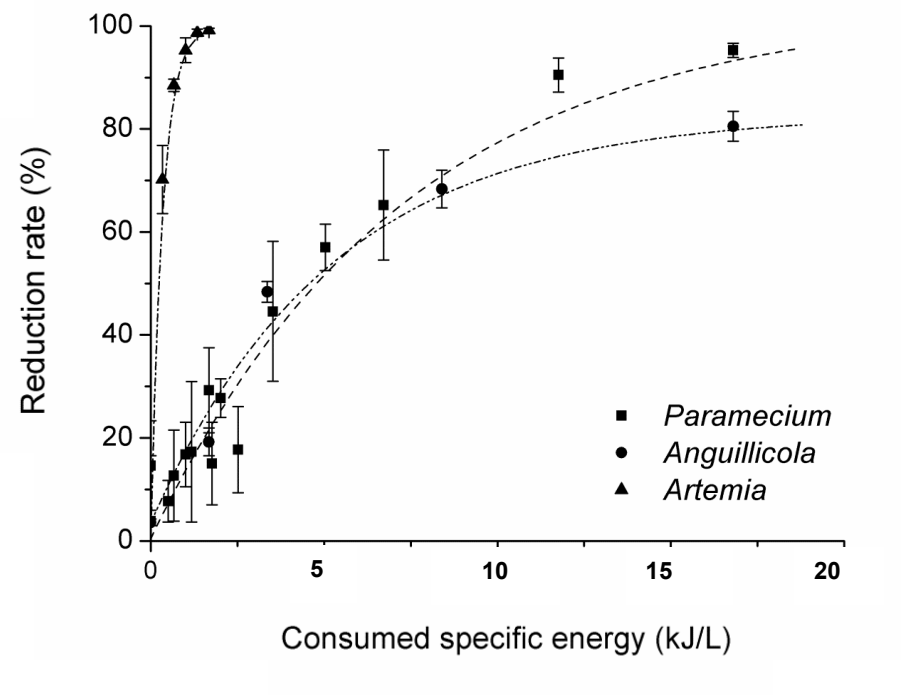

Figure 23. Dose-dependent reduction of *Paramecium* sp., second-stage larvae of the nematode *Anguillicola crassus* and metanauplii of *Artemia* sp. by LFUS. Data are presented as mean ± SD (n = 5). Regression equations and r^2 values are presented in the text.

When *Artemia* sp. was UV-C irradiated following LFUS sonication, the combined treatments had similar reduction trends with a slightly higher efficiency compared to the sole application of LFUS but this increase was negligible (Fig. 24).

Alternating LFUS power density (W/L) and exposure times (s) to add up a constant consumed specific energy of 1.9 kJ/L resulted in constant reduction rate of *Artemia* sp. metanauplii. Only the lowest tested LFUS density (48 W/L) was slightly less efficient in water with low SAC_{254} (SAC_{254} = 27 1/m) ($p < 0.05$) (Fig. 25). Under these conditions, LFUS worked irrespective of UV-C attenuati on properties of the treated water.

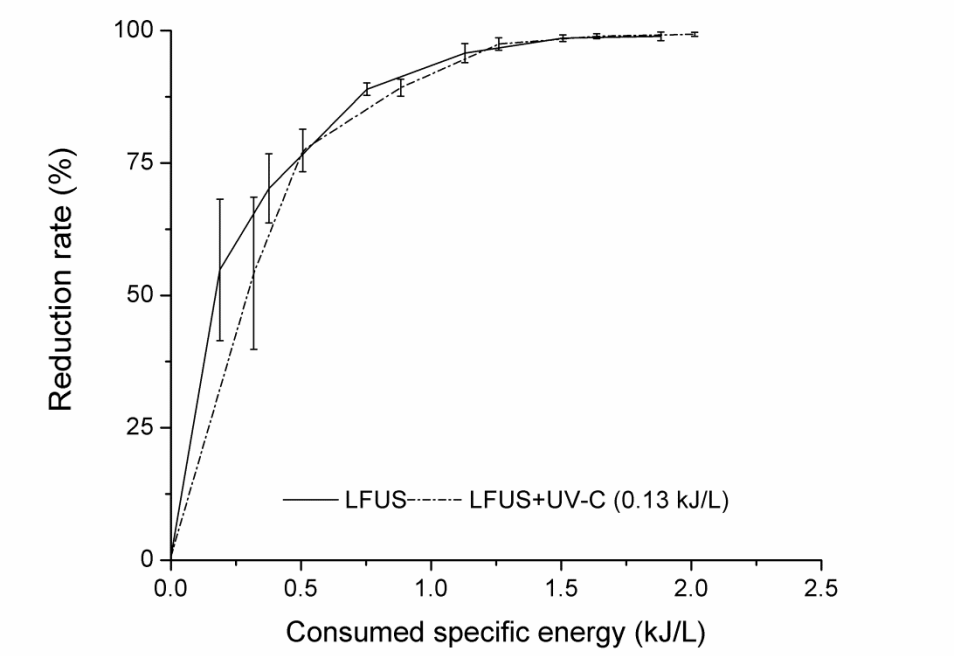

Figure 24. Dose-dependent reduction of *Artemia* sp. by sole application of LFUS and combined with a constant UV-C energy (0.13 kJ/L). Data are presented as mean ± SD (n = 5).

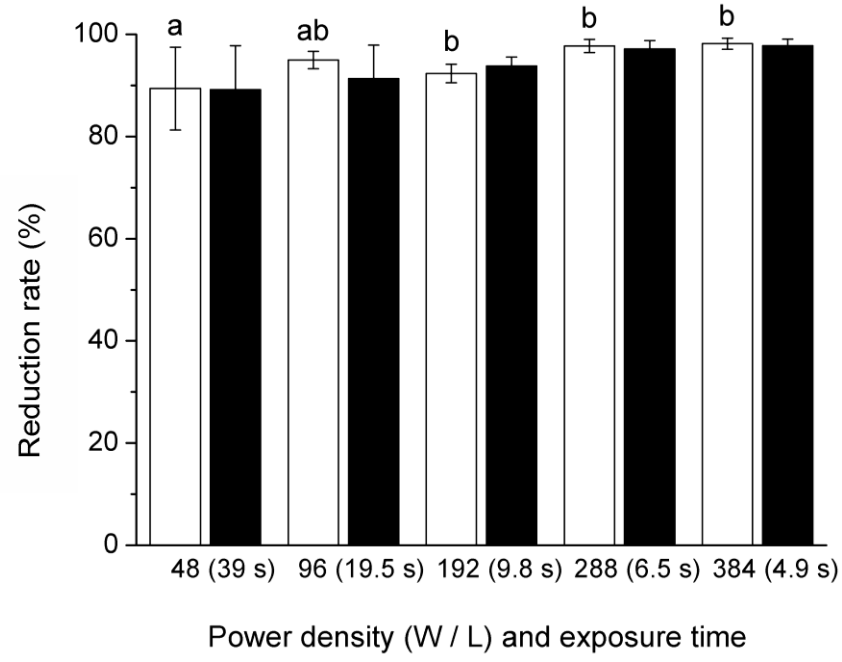

Figure 25. Effect of LFUS applied at constant consumed specific energy (1.9 kJ/L) but variable power on reduction of *Artemia* sp. in water with different spectral attenuation coefficients: SAC_{254} = 27 1/m (**white bars**) and SAC_{254} = 71 1/m (**black bars**). Data are presented as mean ± SD (n = 5), different superscripts denote significant differences between treatments in low SAC_{254} ($p < 0.05$); in high SAC_{254} water reduction rates were not significantly different.

The single-pass reduction rate of free-swimming *Trichodina* sp. in a RAS with the consumed specific energies of 0, 0.8, 1.9, 6 and 19 kJ/L was very similar and matched

to the results for the model organism *Paramecium* sp. that were previously determined by sole LFUS treatment (Fig. 23). By increasing the consumed specific energy of LFUS, the reduction rate of *Trichodina* sp. also increased and reached up to 99 % when the LFUS was operated with consumed specific energy of 19 kJ/L (Fig. 26).The overall reduction rate of pathogenic *Trichodina* sp. compared to the model organism *Paramecium* sp. showed no significant differences in the reduction pattern except the consumed specific energies of 6 kJ/L.

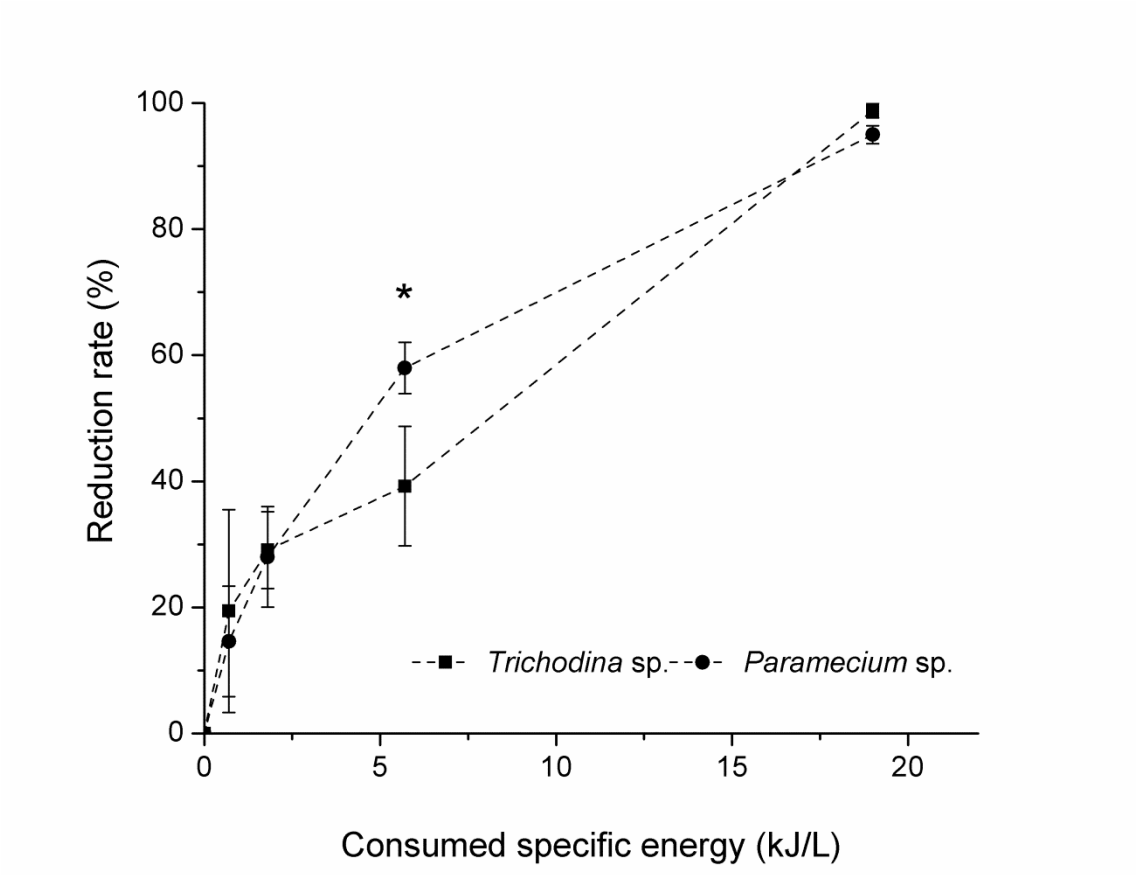

Figure 26. Dose-dependent reduction of *Trichodina* sp. by LFUS (25 kHz) in comparison to *Paramecium* sp.. Data are presented as mean ± SD (n = 5), Asterisk denotes significant difference between the two species ($p < 0.05$).

4.3.2 Continuous-pass mode

The continuous treatment of RAS containing free-swimming *Trichodina* sp. in a bypass mode (38 % RAS water volume per hour) with LFUS (1.9 kJ/L), resulted in a significant reduction of free-swimming *Trichodina* sp. by 94 % within 96 hours. The course of the measured reduction of *Trichodina* sp. followed the theoretical mathematical model, but the measured reduction proceeded more slowly than predicted reduction (Fig. 27).

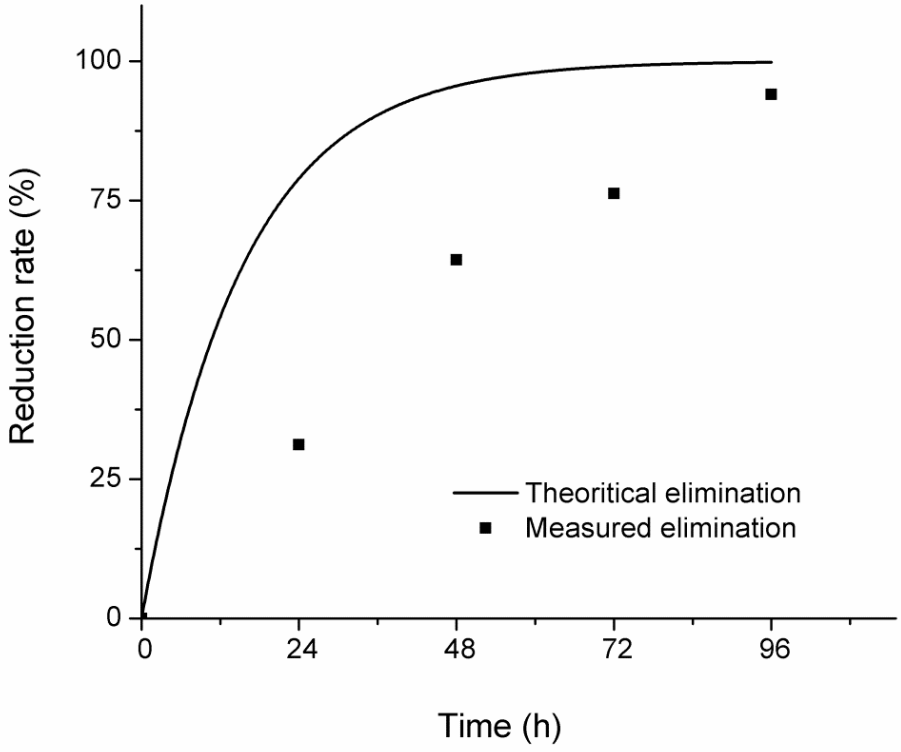

Figure 27. Theoretical and measured reduction of free-swimming *Trichodina* sp. by LFUS (25 kHz) in RAS.

4.4 Photoinduced formation of NO_2^- from NO_3^-

The photoinduced formation of NO_2^- from NO_3^- was correlated with the UV-C dose (rho =0.693, $p \leq 0.001$ at 13 °C and rho = 0.683, $p \leq 0.001$ at 27 °C) and with the NO_3^- concentration (rho =0.484, $p \leq 0.001$ at 13 °C and rho =0.531, $p \leq 0.001$ at 27 °C) (Fig. 28). At a water temperature of 27 °C (Fig. 28 B) the NO_2^- formation was approximately 40 % higher compared to the NO_2^- formation at 13°C (Fig. 28 A). The transformation rate of NO_3^- to NO_2^- showed a positive correlation with the UV-C dose (rho =590, $p \leq 0.001$ at 13 °C and rho = 0.584, $p \leq 0.01$ at 27 °C) and a negative correlation with the NO_3^- concentration (rho =0.267, $p \leq 0.01$ at 13 °C and rho =0.268, $p \leq 0.001$ at 27 °C) (Fig. 29 A and B). At a water temperature of 27 °C , irradiation with 0.042 kJ/L (corresponding to 40 mJ/cm^2 at SAC_{254}= 22.18 1/m), the UV dose recommended for bacterial reduction in drinking water (40 mJ/cm^2), did not lead to a measurable NO_2^- formation at an ambient NO_3^- concentration of 100 mg/L. However, with the same NO_3^- concentration a tenfold higher UV dose of 0.42 kJ/L increased the NO_2^- concentration to above 0.1 mg/L, and a further increase of the UV dose to 6.3 kJ/L resulted in a NO_2^- concentration of 0.6 mg/L. The effect of high NO_3^- concentrations on the NO_2^- yield was relatively small when the irradiation dose was low. At 27 °C,

irradiation of water with a NO_3^- concentration of 1200 mg/L with 0.042 kJ/L resulted in a NO_2^- concentration of 0.05 mg/L. However, the effect of an increased UV dose on the NO_2^- formation was considerably boosted by increased NO_3^- concentrations. For example, at a NO_3^- concentration of 300 mg/L irradiation with 0.42 kJ/L lead to a NO_2^- yield of 0.13 mg/L, and at 1200 mg NO_3^-/L, irradiation with 0.42 kJ/L and 6.3 kJ/L resulted in NO_2^- concentrations of 0.22 and 1.79 mg/L , respectively.

Figure 30 shows the absorbance spectrum of NO_3^-, measured for all NO_3^- concentrations used in this study, with two absorbance peaks: a very strong peak around 200 nm with its shoulder extending beyond 250 nm, and a weak peak around 300 nm.

Accordingly, for the wavelength range around 200 nm there was almost no transmittance across the 5 cm light path, and a depression in the transmission curve at the second peak of the NO_3^- absorbance spectrum around 300 nm. At 253.7 nm, the wavelength mainly responsible for the reduction of microorganisms, NO_3^- concentrations of 100 mg/L and 1200 mg/L reduced the transmittance of the aqueous medium to 31.3 % and 17.9 %, respectively (Fig. 31).

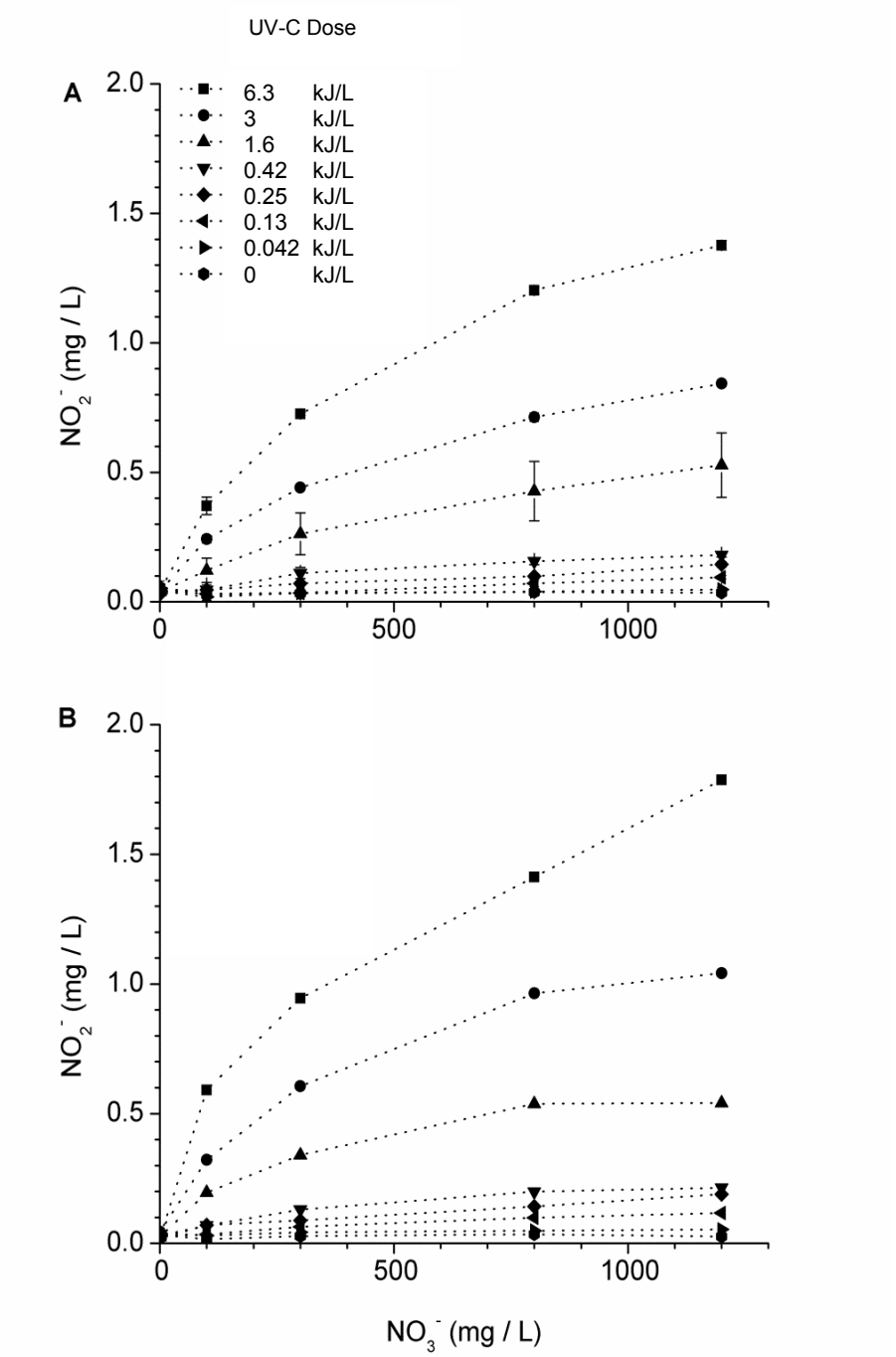

Figure 28. Photoinduced formation of NO_2^- from NO_3^- at different NO_3^-concentrations, UV-C doses and water temperatures, (**A**) 13 °C and (**B**) 27 °C.

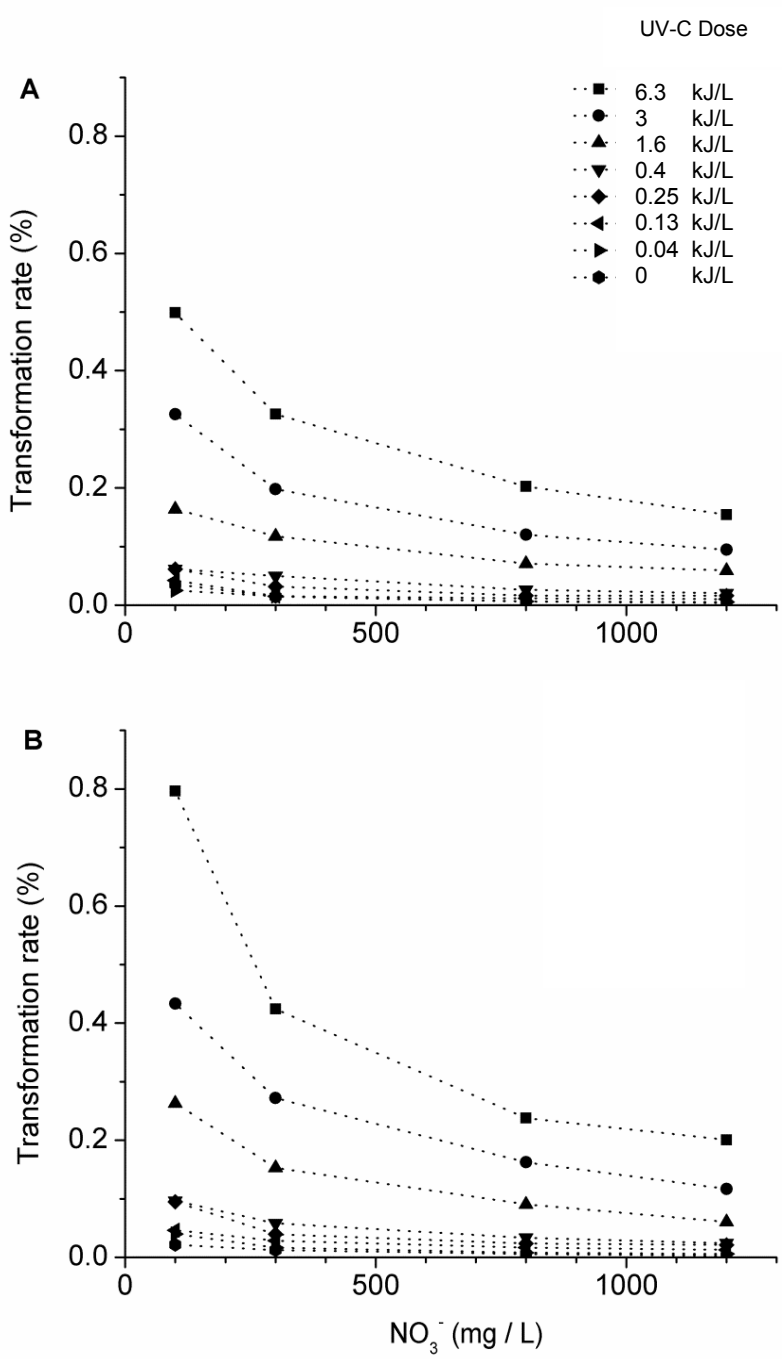

Figure 29. Transformation rate of NO_3^- to NO_2^- at different ambient NO_3^-concentrations, UV-C doses and water temperatures, (**A**) 13 °C and (**B**) 27 °C.

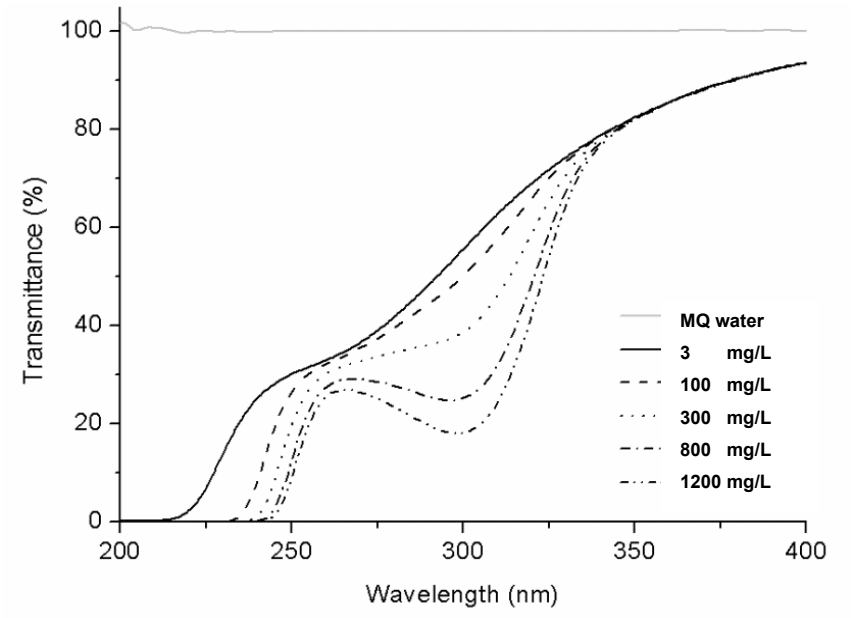

Figure 30. Absorption spectrum of water with different NO_3^- concentrations.

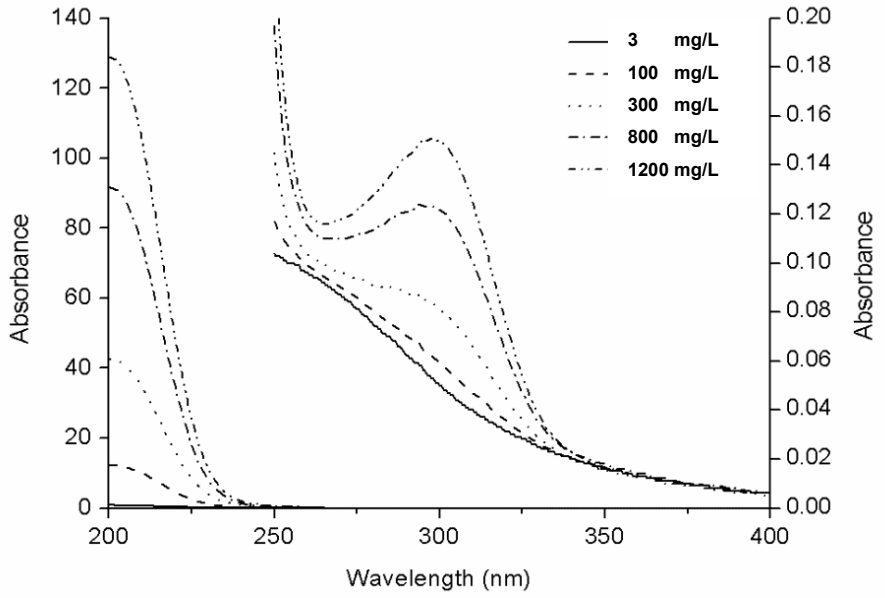

Figure 31. Transmittance spectrum of water with different NO_3^- concentrations, measured in a 5 cm cuvette.

4.5 Ecotoxicological tests

The evaluation of WET by FET and Luminescent bacteria test revealed no evidence of toxic DBPs formation during UV-C irradiation and/or LFUS sonication.

4.5.1 Fish egg test (FET)

FET showed no lethal toxicological endpoints for zebra fish (*Danio rario*) egg after 24 h and 48 h post exposure (Table 3). The positive control 3, 4-Dichloroaniline (3,4-DCA) revealed a 80 % mortality after 48h post exposure.

4.5.2 Luminescent bacteria test

Luminescent bacteria test showed no inhibitory effect on light emission of *Vibrio fischerei* after 30 min post exposure (Table 4). The positive values of reference substances indicated the inhibition of light emission of test luminescent bacteria and reduced viability of the cells. As it is shown in Table 4, the RAS-derived water revealed no positive values that indicated to non-inhibiting effect on the light emission of test luminescent bacteria.

Table 3. Results of fish egg text (FET) for RAS-derived water following UV-C irradiation and/or LFUS.

Treatment	Egg mortality (%)		Coagulation	Formation of somites	Heart-beat	Tail detachment
	24 h	48 h				
Positive control	80	80	yes	no	no	no
Negative control	0	0	No	yes	yes	yes
LFUS (19 kJ/L)	0	0	No	yes	yes	yes
UV-C (1.3 kJ/L)	0	0	No	yes	yes	yes
UV-C (6.3 kJ/L)	0	0	No	yes	yes	yes
LFUS (19 kJ/L) + UV-C (1.3 kJ/L)	0	0	No	yes	yes	yes

Table 4. Results of luminescent bacteria test for RAS-derived water following UV-C irradiation and/or LFUS.

Treatment	Inhibitory effect (%)
Reference substance (Zinc sulfate)	82.1
Reference substance (Potassium dichromate)	46.3
Reference substance (3,5-Dichlorphenol)	33.2
Input water	-9.87
LFUS (19 kJ/L)	-11.37
UV-C (1.3 kJ/L)	-11.9
UV-C (6.3 kJ/L)	-10.7
LFUS (19 kJ/L) +UV-C (1.3 kJ/L)	-10.4

5 Discussion

A healthy and well-functioning aquaculture system can be achieved by sanitary procedures and one of the most important elements in sanitary procedures is disinfection of water against pathogenic microorganisms. Water disinfection is an important issue especially in RAS, which are susceptible to pathogen accumulation and disease outbreaks. The present study is the first comprehensive assessment of LFUS as a novel disinfection technique in RAS.

Pathogenic bacteria, ciliates, nematodes and crustaceans represent major groups for pathogens that are potentially harmful in aquaculture. The model organisms used in the present study were chosen as representatives for each of these groups and cover a vast size range from a few micrometers to half a millimeter. Heterotrophic bacteria comprise important fish pathogens causing bacterial diseases like vibriosis and streptococcosis. The ciliate *Paramecium* sp. was chosen as surrogate for *Ichthyophthiriosis multifillis* and *Trichodina* sp. as common ciliated ectoparasites. *Angullicola crassus*, being a parasite itself, was presumed to be representative of other fish nematodes, for example *Camallanus*. Finally, *Artemia* sp. metanauplii represent larvae of parasitic crustaceans such as *Lernaea*.

5.1 Reduction of bacteria

5.1.1 Single-pass mode

The efficiency of the single-pass modes differed according to the target model organisms. As expected, UV-C irradiation proved to be highly effective against bacteria, but its efficiency was significantly impaired by increasing SAC_{254}. Consequently, the reduction of bacteria caused by a consumed specific energy of 0.13 kJ/L was one to two orders of magnitude higher in water with a relatively low UV-C attenuation (SAC_{254} = 27 1/m) compared to water with a high UV-C attenuation (SAC_{254} = 71 1/m). In this study, bacterial reduction was detected by total viable count. DAPI staining methods showed no significant bacterial reduction effect by single-pass application of UV-C. The UV-C irradiation harms the genomic material of an organism but the naked DNA already persists inside of an intact cell and even in water (Toze 1999). Thus, in contrast to the total viable count, DAPI staining method cannot distinguish between viable and non-viable bacteria and detects the total available bacteria independent of cell viability.

The water with high UV-C attenuation conditions (SAC_{254} = 71 1/m) which we used in this study can be found in RAS with a high stock density and low water exchange. Even under such high UV-C attenuation conditions, total viable counts were still reduced by more than one order of magnitude by consumed specific energy of 0.13 kJ/L.

Treatment with LFUS alone did not prove effective effective reduction in the total viable count within the energy range applied in this study (consumed specific energy consumption up to 19 kJ/L). The successful application of LFUS against bacteria found in previous studies was due to considerably higher energy inputs (Gogate 2007). Holm et al. (2008) for example, working with LFUS of a frequency of 19 kHz, found that a 90 % reduction of different bacteria species requires energy densities ranging from 80 to 1240 kJ/L. Compared to the efficiency of UV-C irradiation in reducing bacteria, even in water with high SAC_{254}, sonication would require at least 600-times the energy input. Irrespective of the technical and financial constraints of implementing such a powerful sonication device for the high flow rates in aquaculture systems, extensive energy input would lead to unacceptable water temperature (Madge and Jensen 2002). Thus, the application of such an enormous ultrasonic energy in order to reduce the bacterial load in RAS will bring an unacceptable temperature increase that is much more than the preferred temperature ranges of fish species.

Particle-associated bacterial communities can represent a significant proportion of total bacteria in the water (Simon et al. 2002). The particulate matter of water as a shelter protect bacteria from external stressors such as UV-C irradiation (Parker and Darby 1995; Tang et al. 2011). In the present study, de-agglomeration of particle-associated bacterial communities by LFUS improved the efficiency of UV-C against bacteria up to 0.6 log units compared to sole application of UV-C with the same dose of 1.3 kJ/L (consumed specific energy). Similarly, Blume and Neis (2004) have shown an improved reduction of *Escherichia coli* and fecal streptococci in wastewater by 0.8 log units applying pretreatment with LFUS (20 kHz) followed by UV-C irradiation with 1.5 kJ/L (consumed specific energy). However, in addition to this positive effect, a reduced mean particle size might also impair the mechanical removability of suspended solids in physical filters such as disc and drum filters used in RAS. This possible side effect of LFUS needs further attention.

5.1.2 Continuous-pass mode

Assuming a mean generation time of 2.7 h for free viable heterotrophic bacteria (Leonard et al. 2000) and a reduction by only one log unit, the mathematical model

predicts that the bacterial count in the RAS could be reduced by 90 % within 2 days when the UV-C disinfection reactor is operated with at least 30 % of RAS water volume per hour. However, our empiric results showed that the bacterial growth overcompensates the reduction by UV-C irradiation even when the UV-C disinfection reactor is operated in bypass with 67 % of the RAS water volume per hour. The result of bypass application of UV-C indicated that the reduction of heterotrophic bacteria requires a high turnover rate in order to compensate their short generation time. In this study, only the full-flow application of the UV-C disinfection reactor efficiently reduced the number of heterotrophic bacteria in the circulating water of RAS. During the experimental period (4 days) the UV-C disinfection reactor achieved a bacterial reduction by only one log unit and we could not reach to a higher bacterial reduction rate. Despite the significant reduction of bacterial count by continuous application of UV-C in full-flow mode, the course of the measured bacterial reduction was much slower than the prediction by the mathematical model. RAS similar to other aquatic ecosystems contain heterogeneous bacterial communities composed by bacteria with different growth rates and generation times. In our mathematical model we assumed the mean generation time of 2.7 h (Leonard et al. 2000) that was within the normal range for microbial communities observed in biological treatment of wastewater (Pollard and Grennfield 1997). A possible explanation of the difference between the predicted and measured bacterial reduction could be due to the faster growth and shorter generation time of the heterotrophic bacterial communities in our experimental RAS.

Due to the importance of the particulate matters in RAS and their role as refuge for bacteria, the complete reduction (100 %) of heterotrophic bacteria can be difficult. Therefore, sole application of UV-C even with high doses cannot inactivate the embedded bacteria in particles and these bacteria will remain intact. Therefore sonication of circulating water in RAS with LFUS will be an alternative to reach a higher bacterial reduction compared to sole application of UV-C.

However, in our study the treatment of RAS water with combined disinfection reactor when a 110 W UV-C disinfection reactor was operated following a pretreatment with LFUS (1.4 kW) in a bypass with 25 % of RAS water volume per hour did not show a bacterial reduction compared to control RAS. The explanation of these results probably could be due to in the low water flow capacity of our combined LFUS/UV-C reactor. According to the technical specification, the maximum allowed flow rate was 3 m^3/h equal to 25 % of RAS water volume per hour which probably was not enough for

having an efficient de-agglomeration effect by LFUS on particulate matters in circulating water. However, the mathematical model shows that the most crucial factor for an effective reduction of the bacterial load by means of a flow-through reactor is the balance between flow rate and the mean bacterial generation time.

5.2 Reduction of eukaryotic organisms

5.2.1 Single-pass mode

Reduction of eukaryotic model organisms in our study ciliates, nematodes, and crustaceans, required a much higher UV-C dose compared to the reduction of bacteria. Even the highest UV-C dose we could realize in our experiments, being ten-fold higher than the dose reducing the CFU by 2.3 log units, only reduced approximately 80 % of the crustacean and nematode larvae, and 40 % of the ciliates. Similarly, reduction of therons of *Ichthyophthirius multifiilis* and *cryptocaryon irritans* as ciliated ectoparasites needed approximately 3 times (Gratzek et al. 1983; Hoffmann 1974) and 20 times (Colorni and Burgess 1997) more UV-C dose, than the recommended UV-C dose of 40 mWs/cm^2 for reduction of bacteria.

LFUS proved to be effective against each of the three eukaryotic model organisms, but the efficiency of sonication differed greatly between species with different size. *Artemia* sp. as the largest target organism with a body length of 500 - 700 µm was the most affected by LFUS. A dose of 1.9 kJ/L was sufficient to achieve a 99 % reduction of the *Artemia* sp.

Compared to ciliates with a cell length of 70 – 110 µm, *Artemia* sp. required only one twentieth of the LFUS dose to obtain a reduction rate of 99 %. Holm *et al*. (2008) also found an inverse relationship between LFUS treatment efficiency and the size of organism. Accordingly, Wolber and Pietrock (2004) identified a LFUS dose of 6.3 kJ/L to kill 100 % of cercaria of *Bucephalus polymorphus* that have a body length of about 200 µm.

Artemia sp. has already been used as model organism to evaluate LFUS as a treatment option for ballast water (Holm et al. 2008; Sassi et al. 2005). Using a laboratory-scale flow-through device consisting of a beaker and a rod-shaped sonotrode operating at a frequency of 19 kHz, Holm et al. (2008) determined an energy consumption of 8 kJ/L to obtain a 90 % reduction of *Artemia* sp. nauplii. Sassi et al. (2005) used a similar, but larger device with a rod-shaped sonotrode mounted inside a steel box serving as flow-

through device. With a frequency of 20 kHz they determined an energy consumption of 18 kJ/L for a 100 % reduction of *Artemia* sp. nauplii. In the present study, comparable effects were obtained with ten-time lower energy consumption. These results show that technical optimization can considerably improve the operating efficiency of LFUS for applications requiring high flow rates.

In this study we examined the effect of different SAC_{254} and the constant applied energy, but variable power against *Artemia* sp. Only the reduction rates determined for the lowest power density (48 W/L) showed a slightly reduced efficiency. Thus, the destructive effect of LFUS depended on the applied volume-specific energy that can be realized by any constant product of the specific power and retention time. The findings of this aspect of the experiment might be helpful for further adjustment of LFUS disinfection reactors, since specific applications have different needs concerning the reduction rates of certain species, as well as the technically feasible flow rate and power.

The results of this study proved that LFUS worked irrespective of quality (expressed as SAC_{254}) of the treated water. This finding is in accordance with Madge and Jense, (2002) who showed that water quality does not significantly affect the disinfection efficiency of LFUS.

The similarity of the dose-effect relationships of LFUS for the reduction of *Trichodina* sp. and *Paramecium* sp. showed that reduction of similar sized ciliates will occur irrespective of the species, and confirmed the suitability of *Paramecium* sp. as model organism for ciliated ectoparasites. The size and morphology of the target organisms likely played an important role in disinfection efficiency of LFUS. Accordingly, Thacker (1973) found that the size and shape of cells, as well as the tensile strength of cell walls or membranes is important in the use of ultrasound for cell disruption.

Comparing the energetic efficiency of LFUS and UV-C for 40 % reduction of eukaryotic model organisms, LFUS proved to be five times more efficient against *Artemia* sp. than UV-C, whilst UV-C was three and even nine times more efficient against *Paramecium* sp. and *Anguillicola crassus* larvae, respectively. However, comparing the energy efficiency of LFUS sonication and UV-C irradiation, it must be considered that the efficiency of a UV-C device depended on the SAC_{254} of the treated water, the construction of the disinfection reactor and type of UV-C lamp. For the UV-C disinfection reactor used in our study, increasing the SAC_{254} value from 22 1/m to 70 1/m increases the energy demand by factor 5.5 obtaining the same effective UV-C dose

(Micro light Basic 5; a.c.k. aqua concept, Karlsruhe, Germany). Furthermore, the gap width of the reaction chamber was crucial, especially at high SAC_{254} values, as attenuation increased exponentially with the gap width. Realization of high power densities with medium pressure lamps would decrease the UV-C efficiency by factor 3 because their germicidal UV efficiency is only one third that of a low pressure mercury lamp (U.S.EPA 2006). Under such conditions, the efficiency of LFUS and UV-C against ciliates or nematode larvae will be in a similar range, and under most unfavorable conditions for UV-C, application of LFUS would be the most efficient treatment. In contrast to UV-C, LFUS is robust against different water quality (Madge and Jensen 2002) and can be applied at energy densities that are effective against a wide range of parasites like ciliates, nematodes and crustaceans. Thus, a combination of UV-C (against viruses and bacteria) and LFUS (against eukaryotic parasites) could provide an appropriate water treatment concerning all relevant pathogens in RAS.

5.2.2 Continuous-pass mode

A simple mathematical model based on the assumption of exponential growth and linear decrease, can be used to estimate the least reduction rate and flow rate that are required to achieve reduction of a certain organism in a RAS. However, our model used assumptions that were not fully met in the reality. The most critical points are that the pathogens would not show an exponential growth under limiting conditions, and they were not uniformly distributed in the water, but they also live in or on the fish. Thus, the model was suitable to get an idea about the feasibility of a certain treatment, but finally the process parameters must be determined by empirical data.

In accordance to the prediction based on the mathematical model, free-swimming *Trichodina* sp. were efficiently reduced by LFUS operated in a bypass mode. However, the measured reduction was slower compared to the theoretical prediction. This observation can be easily explained by the fact that the parasites are not only free-swimming in the water as assumed in the model, but of course also live on the fish as their host.

5.3 Photoinduced formation of NO_2^- from NO_3^-

The results of this study as expected showed that the NO_2^- yield increased with increasing UV-C doses, increasing NO_3^- concentrations and increasing water temperature. Similarly, the transformation rate of NO_3^- to NO_2^- was positively correlated with an increase of the UV-C dose, but negatively correlated with an increase of NO_3^- concentrations. A likely explanation for the latter condition can be found in the subsequent reactions during NO_3^- photolysis (Mack and Bolton 1999; Wagner et al. 1980). The intermediate NO_2• radical can react with the •OH radical to NO_3^- (Mack and Bolton 1999). The back transformation to NO_3^- increases with the NO_2• concentration and finally inhibits the NO_2^- production (Wagner et al. 1980; Mack and Bolton 1999). Parts of this chain reaction have considerably slow reaction rates and thus, with a too short time-frame, the chain of reactions mentioned above does not reach equilibrium between NO_3^- and NO_2^- molecules (Wagner et al. 1980). Furthermore, the high optical densities of the NO_3^- solutions used in this study resulted in an inhomogeneous exposure of NO_3^- to UV light within the disinfection reactor. Consequently, not all potentially transformable NO_3^- could be transformed to NO_2^- (Wagner et al. 1980). Because the treatments with nitrate concentrations > 100 mg/L used in our study were optically very dense in the UV range and the residence time of the water in the UV-C disinfection reactor was relatively short, this, together with the self-inhibition of the nitrite production, has likely led to the lower nitrate transformation rates at higher nitrate concentrations.

When UV-C irradiation is used to improve the rearing conditions for fish in aquaculture or in aquaria, there are several scenarios in which the photoinduced formation of NO_2^- by LP lamps is a matter of concern: When UV doses exceed by far those regularly used for disinfection; the NO_2^- concentrations in the outflow of the UV disinfection reactor are significantly increased. This can be the case when greatly oversized UV units are operated in relatively small systems such as aquaria, or when UV irradiation is used to control eukaryotic pathogens, which requires considerable more energy compared to bacterial reduction.

In addition, high ambient NO_3^- levels promote the photoinduced NO_2^- formation. Consequently, a high UV dose to remove eukaryotic pathogens in combination with high NO_3^- levels of several 100 mg that are typical for intensive RAS can lead to a significantly increased NO_2^- load in the outflow of the UV disinfection reactor even when a LP-lamp is used. In RAS or aquaria with a well-functioning biofilter, nitrifying

bacteria will quickly transform the NO_2^- formed by photolysis of NO_3^- back to NO_3^-. However, it should be considered that UV irradiation is commonly used as the final step of the water treatment, and thus fish are continuously exposed to the NO_2^- concentration that leaves the UV disinfection reactor. In systems without a well-functioning biofilter, NO_2^- can accumulate, and depending on UV dose, recirculation time, and total water volume of the system, NO_2^- can rise to a critical concentration within a short time.

Whether a certain NO_2^- concentration is critically harmful for the cultured fish cannot easily be answered. Fish species differ greatly in their susceptibility to NO_2^- (Lewis et al. 1986; Kroupova et al. 2005; Wuertz et al. 2013), and NO_2^- toxicity depends on many factors (Kroupova et al. 2005). The presence of chloride (Cl^-) especially significantly decreases NO_2^- toxicity because both ions compete for the branchial Cl^- uptake mechanism (Jensen 2003; Kroupova et al. 2005; Tomasso and Grosell 2005; Wuertz et al. 2013). Published acute toxicity data for NO_2^-, measured as LC50 values, ranged from 0.6 to 2.9 mg/L for salmonids to 460 mg/L for largemouth bass, *Micropterus salmoides* (Wuertz et al. 2013). Thus, under common conditions, fish mortality due to the photoinduced NO_2^- formation by a LP lamp is unlikely, when biofilters are employed. However, if, e.g., water with a NO_3^- concentration of 300 mg/L is irradiated with the UV dose of 1.6 kJ/L (equivalent to 1600 mJ/cm^2 for SAC_{254} = 22.18 1/m, 40 times more than the dose recommended for the treatment of drinking water), a single flow-through the UV-C disinfection reactor could already lead to a NO_2^- concentration that are lethal for some susceptible species such as salmonids.

In addition to the acute toxicity, sublethal effects of NO_2^- on the fish must be considered. Subchronic intoxication with NO_2^- concentrations far below the LD50 value can cause physiological disturbances, tissue damage and reduced growth (Alcaraz and Espina 1997; Frances et al. 1998; Kroupova et al. 2008; Wuertz et al. 2013). Little is known about safe NO_2^- concentrations that cause no pathological changes in different fish species. For rainbow trout, *Oncorhynchus mykiss*, a representative of the most susceptible fish species, Kroupova et al. (2008) estimated a NOEC (28d LC_0) at 0.01 mg/L NO_2^- the basis of growth rate inhibition. For pike-perch, *Sander lucioperca*, a moderately sensitive species, Wuertz et al. (2013) considered a NO_2^- concentration < 0.2 mg/L as safe. Therefore, it is a good idea to keep the NO_2^- concentration in the fish tanks as low as possible both from animal welfare as well as economic considerations. Although LP lamps are commonly assumed to be harmless in terms of the formation of by-products, the potential of low-pressure UV lamp irradiation to

induce photolysis of NO_3^- to NO_2^- should be carefully considered; especially when high levels of NO_3^- are present and high UV doses are to be applied. The potential to increase the UV-C dose against eukaryotic parasites is limited by the photoinduced reduction of nitrate to nitrite, then the synergistic application of UV with another disinfection strategy such as LFUS, as it was already suggested for ballast water treatment (Sassi et al. 2005), may be a good alternative for aquaculture purposes.

5.4 Ecotoxicological tests

With respect to possible DBPs, both ecotoxicological tests showed no WET and proved the safe application of the novel LFUS/UV-C disinfection strategy, even when UV-C or LFUS were applied with the highest consumed specific energies we could realize with our equipment. However, it should be noted that all our experiments were conducted with a LP-Lamp, and when using a MP-Lamp, due to the different emission spectrum, other results may emerge. In the present study we used zebrefish egg test and luminoset bacteria tests which are routine and common ecotoxicological tests for the evaluation of WET in waste and drinking water systems. Neither tests detected the problem of photoinduced formation of nitrite from nitrate. This might be explained by a certain nitrite tolerance of the test organisms. The toxicity of nitrite to bacteria is attributed to NO (Zhang et al. 2013), and *V. fischeri* uses an alternative oxidase, which plays a role in NO resistance (Dunn et al. 2010). Concerning the FET, it was shown that zebrafish eggs possess a high nitrite tolerance. (Meinelt et al. 2010; Simmons et al. 2012).

6 Conclusion

In RAS, UV-C light irradiation by using low pressure lamps with low energy consumption is a suitable disinfection strategy for reducing heterotrophic bacteria. In RAS with high nitrate content, the reduction of eukaryotic parasites by increasing the UV-C dose, much higher than the recommended dose against bacteria, might be limited by the photoinduced formation of nitrite from nitrate.

The application of LFUS might then be a suitable and safe-applicable disinfection method for the reduction of eukaryotic parasites and can thus be a useful supplementation to UV-C used against bacteria.

In practice, a combined LFUS/UV-C disinfection reactor can be applied in RAS. In a combined reactor, the UV-unit determines the necessary flow rate. Thus, in smaller RAS this reactor should be operated continuously with the full-flow of the system and would be installed directly in the filter circuit. At high flow rates, it may make more sense from a technical and energetic point of view to use separate UV-C and LFUS disinfection reactors. In this situation UV-C reactor with typically recommended UV-C dose agaist bacteria should be operated in the full volumetric flow of the system and LFUS can then be operated in the bypass mode for the reduction of eukaryotic parasites.

This study showed that the combination of LFUS and UV-C could provide an appropriate water disinfection with regards to all relevant pathogens in recirculating aquaculture systems.

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Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe. Des Weiteren erkläre ich meine Kenntnisnahme der dem angestrebten Verfahren zugrunde liegenden Promotionsordnung. Ich habe mich anderweitig nicht um einen Doktorgrad beworben und bin nicht im Besitz eines entsprechenden Doktorgrads.

Berlin, 30.09.2014
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